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METABOLISM OF RADIOACTIVE SILVER IN A PATIENT WITH CARCINOID

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Carcinoid tissue is argyrophyllic by staining techniques. Adrenal tissue of rats, which is also argyrophyllic, has been shown to take up the highest percentage per gram of tissue of intravenously administered radiosilver. For diagnostic and possibly later therapeutic purposes, radioactive silver was given intravenously to a patient with functioning malignant carcinoid, after incubation with his own blood. The carcinoid tissue showed a low uptake, but the metabolic pathways of radiosilver in man are being reported. It was at first mostly associated with the red blood cell, probably because of the incubation, and in the plasma with the globulin fraction. The radiosilver was rapidly removed from the blood, apparently by the liver. At postmortem, it was found mainly in the liver and skin, but because of its relative weight the highest accumulation was in the skin. Excretion of the radiosilver was principally in the feces, presumably via the bile into the gastrointestinal tract. A slight amount of radioactive silver was excreted in the urine, probably as a function of plasma concentration.

IT IS well known that carcinoid tissue is argyrophyllic by staining techniques. The possibility that this type of tumor might differentially accumulate radioactive silver prompted its use as a tracer, and indicated the possible later use of beta emitting silver for therapeutic purposes. The difference between in vitro staining and in vivo concentration was fully appreciated, but in Scott and

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Hamilton's[1] study of rats the adrenal glands, which are also highly argyrophyllic by in vitro staining, took up the highest percentage per gram of tissue when carrier-free radiosilver was given intravenously. West and Goldie[2] noted that the major portion of insoluble silver^{111} oxide, when injected subcutaneously in the vicinity, was taken up by malignant tumors of mice. Although the results showed little accumulation of silver in our patient with functioning malignant carcinoid, the metabolic pathways of silver in man by use of intravenous radiosilver as a tracer have, to our knowledge, not been reported.

The use of radioactive silver as a tracer in animals has not been extensive. Scott and Hamilton[1, 3] studied the distribution of soluble radiosilver in various tissues of the rat. West and co-workers[4, 5] noted that radioactive silver^{111} concentrated in areas of infection in rats.

For initial tracer use, silver^{106} was selected because of its gamma emission and half-life of 8.2 days, and was available with other radiosilvers admixed. The longer-lived isotopes proved very useful for postmortem studies.

METHODS

Approximately 43 μc cyclotron produced radioactive silver that contained less than 0.1 mg. of carrier silver was used. From decay measurements and the supplier's information, the activity was calculated as distributed among the silver isotopes as follows: 15 μc Ag^{105}, 40 day half-life; 15 μc Ag^{106}, 8 day half-life; 5 μc Ag^{110m}, 270 day half-life; and 8 μc Ag^{111}, 7.5 day half-life. The silver was transferred to a silicone coated bottle that contained 10 ml. acid citrate dextrose solution to which 45 ml. of the patient's blood had been added. This was shaken gently during incubation at room temperature for 30 minutes, after which 50 ml. was given intravenously to the patient.

Heparinized blood samples were obtained at frequent intervals during the first day, and variously for the next 3 weeks. Whole blood and plasma samples were compared in a scintillation well counter to appropriate standards. Pooled plasma was separated with 20 and 50 per cent ammonium sulfate solution into fibrinogen, globulin, and albumin fractions, and the radioactivity was counted. Frequent external counts over the heart, head, liver, spleen, adrenals, abdomen, sacrum, feet, and contralateral control areas were performed with a collimated scintillation counter during the first day, and at frequent intervals for the next 3½ months. Twenty-four hour stool and urine collections were made for the first 3 days, periodically during the next 3 weeks, and the radioactivity was compared with a standard. The patient died after 195 days and postmortem tissue specimens were taken from available organs. The tissues were weighed, digested with concentrated nitric acid, treated with isoamyl alcohol or 2:1 alcohol-ether to remove fat, dissolved in 1,4-dioxane, and the radioactivity was determined in a scintillation well counter.

RESULTS

Blood Transport.—Table I shows the percentage of radioactive silver per 100 ml. whole blood and plasma at various intervals after the injection. At the time of first blood sampling, 7 minutes after injection, only 0.74 per cent of the injected dose per 100 ml. of blood remained. At an assumed blood volume of 7 per cent of body weight (58 kilograms), this represents approximately 30 per cent of the injected dose in the circulation. At 2 hours 10 per cent remained and at one day 2 per cent, continuing at approximately this level for at least 20 days. During the first few days, the per cent of radioactive silver was higher in whole blood than plasma and by calculation, a larger proportion was attached

TABLE I. RADIOACTIVE SILVER IN WHOLE BLOOD, PLASMA, AND RED BLOOD CELLS

TIME	% OF DOSE PER 100 ML.	
	WHOLE BLOOD	PLASMA
7 min.	0.74	0.46
20 min.	0.57	0.39
30 min.	0.49	0.33
40 min.	0.43	0.32
50 min.	0.40	0.23
1 hr.	0.35	0.23
1½ hr.	0.29	0.15
2 hr.	0.22	0.11
3 hr.	0.16	0.081
4 hr.	0.13	0.067
5 hr.	0.10	0.068
6 hr.	0.090	0.056
7 hr.	0.081	0.053
1 day	0.051	0.040
2 day	0.046	0.039
3 day	0.043	0.040
6 day	0.043	0.046
8 day	0.046	0.043
13 day	0.046	0.031
20 day	0.035	0.041

to the red blood cells than was transported in the plasma. The total recovery in the various fractions of the pooled plasma represents 87 per cent of the original pooled plasma counts; 77 per cent of the radioactive silver in the plasma is carried in the globulin fraction, 15 per cent in the albumin, and 8 per cent in the fibrinogen fractions.

External Counting.—Counting rates obtained over various areas of the body, corrected for physical decay, were plotted logarithmically against time. For the liver, Fig. 1, this resulted in a straight line representing a biologic decay of 48 day half-life. The curves, Figs. 2, 3, and 4, obtained by counting over other areas, however, could be resolved into two exponential decay components. The short half-life components were obtained by subtracting the extrapolated 48 day half-life values from the corresponding values of the experimental curve. The short half-life decay components had half-lives of approximately 3½ days. Comparison of the extrapolated zero-time values of the two components shows the short half-life component to be approximately 30 per cent of total activity over the chest (Fig. 2), 40 per cent over the sacrum (Fig. 3), and a maximum 50 per cent over the heart (Fig. 4).

TABLE II. RADIOACTIVE SILVER IN STOOL AND URINE

DAY	% OF DOSE	
	STOOL*	URINE
0-1	2.8	0.29
1-2	2.9	0.11
2-3	1.8	0.07
3-6	3.8 (1.3†)	0.15 (0.05†)
7-8	1.2	0.07
12-13	0.49	0.03
20-21	1.33	0.04

*Includes colostomy collection.
†Mean average per day.

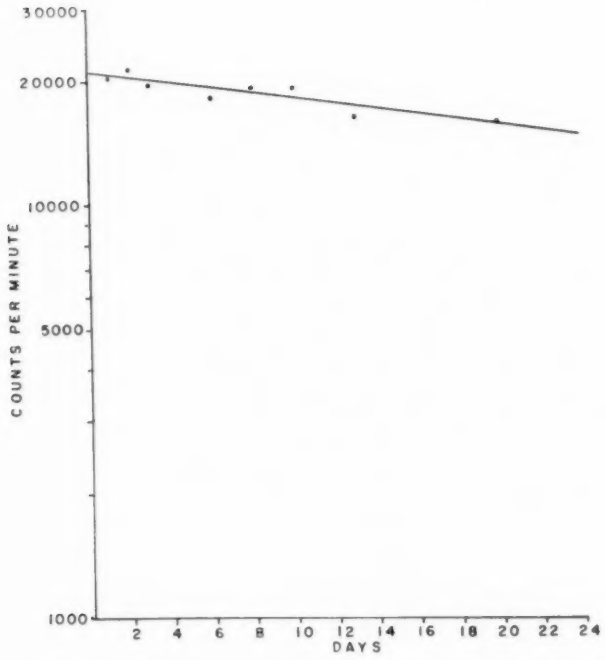

Fig. 1.—External counting—liver. Radiosilver biologic half-life of 48 days.

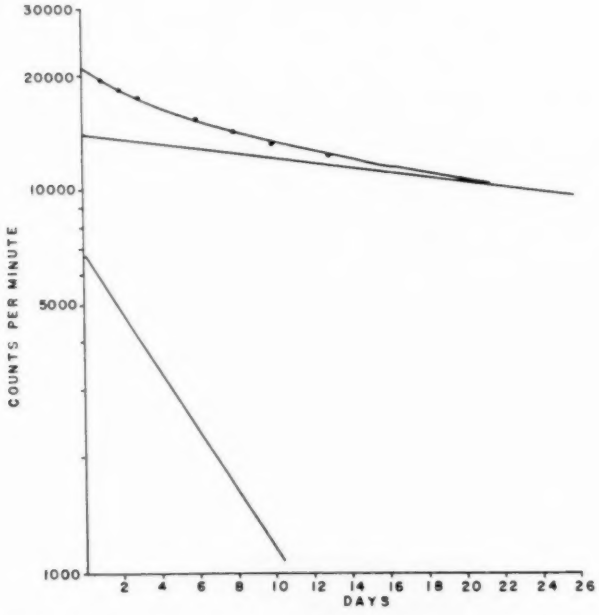

Fig. 2.—External counting—chest. Radiosilver biologic half-lives of 48 and 3.8 days.

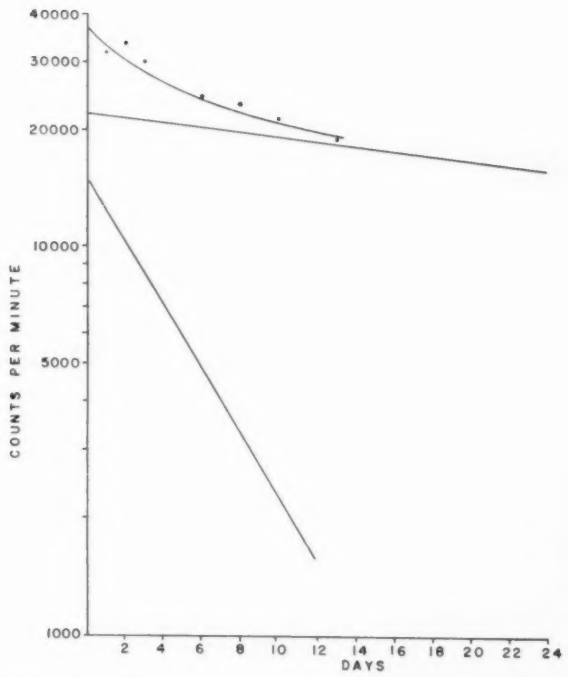

Fig. 3.—External counting—sacrum. Radiosilver biologic half-lives of 48 and 3.6 days.

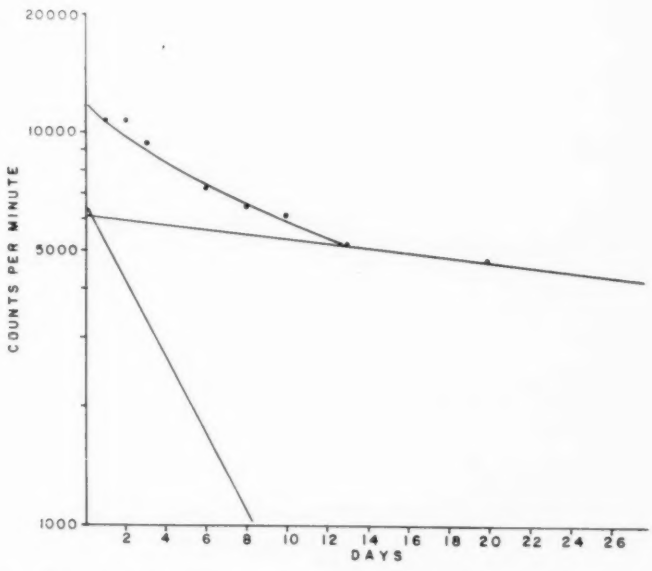

Fig. 4.—External counting—heart. Radiosilver biologic half-lives of 48 and 3.2 days.

Excretion.—Table II shows that elimination of radiosilver from the body is mainly via the feces. Except for the first 2 days with a slightly higher rate, there seems to be a comparatively constant rate of excretion. Urinary excretion amounted to only 5 per cent of the fecal excretion. When the per cent of silver in the plasma, Table I, is compared with the urinary excretion, Table II, it appears that the urinary excretion is a direct function of the plasma content of silver.

TABLE III. RADIOACTIVE SILVER IN TISSUES*

ORGAN	C.P.M./GM.	ORGAN	C.P.M./GM.
Liver	70.0	Prostate	3.4
Skin	37.7	Heart	3.1
Kidney, left	10.0	Stomach	3.0
Brain	9.2	Rib	2.5
Kidney, right	7.5	Ileum	2.2
Lymph node, abdominal	5.6	Carcinoid, liver (metastatic)	1.5
Adrenal gland	5.5	Lung	1.4
Carcinoid, ileum (primary)	5.1	Muscle	1.0
Testis	5.0	Spleen	0.7
Aorta	4.1	Thyroid	0.0
Pancreas	4.0	Ascitic fluid	0.0
Urinary bladder	3.8		

*Postmortem, 195 days after injection of 43 μc.

Postmortem Findings.—Table III shows the relative content of radiosilver in the various organs at postmortem examination, in counts per minute per gram of tissue. The highest accumulation was found in the liver. The metastatic carcinoid tissue within the liver showed extremely low counts, even lower than in the primary tumor. Other organs that contained significant amounts of silver were the skin, brain, and kidneys.

DISCUSSION

In our experiment the total amount of carrier silver was below 0.1 mg., or 0.002 mg. per kilogram of man, and the behavior should therefore correspond closely with that of carrier-free radiosilver administration used by Scott and Hamilton[2] in the rat. The rapid removal of radioactive silver from the blood is consistent with their findings of 86 per cent radiosilver in the liver of the rat 5 minutes after intravenous injection. The radiosilver associated with the red blood cell is much higher in our studies with a human, but our method included incubation with the blood for 30 minutes. The proportions found in the plasma grossly correspond to their values; 85 per cent against their 80 per cent in the globulin and fibrinogen fractions, and 15 per cent against their 20 per cent for the albumin fraction.

A possible explanation for the short half-life component of biologic decay found by external counting is that it represents silver bound to the red blood cells. It is thus greatest in the heart, and the red blood cells, in the early phase, show greater specific activity than the plasma. The long half-life component of biologic decay is seen principally in the liver, which is probably the main site of long-term storage, as well as the excretory organ.

In view of the high external counts over the liver, and later postmortem findings, it would appear that radiosilver is passed into the gastrointestinal tract via the bile, and later excreted in the stools. In our studies, the rate of excretion was rather slow, occurring over a period of weeks, as compared to the animal studies, where 94 per cent was excreted in 4 days.

From the postmortem studies, it is obvious that the radiosilver was retained in the body for a long period of time, chiefly in the liver and skin. It is interesting that the clinical syndrome of argyria[6] is due to deposition of absorbed silver in the skin, and the skin contained the second highest amount per gram of tissue, and because of its relative weight, the highest accumulation.

The lack of high activity of silver in the carcinoid tissue was disappointing. The lower counts in the metastatic carcinoid of the liver than in the primary tumor may be due to more necrosis, or the proximity of the liver as the chief excretory organ.

SUMMARY

1. Radioactive silver, after incubation with his own blood, was given intravenously to a patient with functioning malignant carcinoid.

2. The radiosilver was at first mostly associated with the red blood cell, probably because of the incubation, and in the plasma with the globulin fraction.

3. The radiosilver was rapidly removed from the blood apparently by the liver with a biologic half-time of 48 days. At postmortem, it was found mainly in the liver and skin in the approximate ratio of two to one per gram of tissue, but because of its relative weight, the highest accumulation was in the skin. The carcinoid tissue showed a low amount of silver.

4. Excretion of the radiosilver was principally in the feces, presumably via the bile into the gastrointestinal tract, occurring slowly and almost at a constant rate over a long period of time. A slight amount of radio-silver was excreted in the urine, probably as a function of plasma concentration.

REFERENCES

1. Scott, K. G., and Hamilton, J. G.: The Metabolism of Silver in the Rat With Radio-Silver Used as an Indicator, Univ. California Pub. Pharmacol. 2: 241, 1950.
2. West, H. D., and Goldie, H.: Topical Localization in Mouse of Radioactive Silver Oxide $(Ag^{111})_2O$ Introduced by Various Routes, Proc. Soc. Exper. Biol. & Med. 92: 116, 1956.
3. Scott, K. G., and Hamilton, J. G.: The Metabolism of Silver, J. Clin. Invest. 27: 555, 1948.
4. West, H. D., Johnson, A. P., and Johnson, C. W.: The Use of Radioactive Silver for the Detection of Abscesses and Tumors. I. The Concentration of Ag^{111} in Spontaneous and Experimentally Induced Abscesses, J. Lab. & Clin. Med. 34: 1376, 1949.
5. West, H. D., Elliott, R. R., Johnson, A. P., and Johnson, C. W.: In Vivo Localization of Radioactive Silver at Predetermined Sites in Tissue, Am. J. Roentgenol. 64: 831, 1950.
6. Gill, W. R., and Pillsbury, D. M.: Argyria. The Pharmacology of Silver, Baltimore, 1939, Williams & Wilkins Company.

CATECHOLAMINES IN PATIENTS WITH PHEOCHROMOCYTOMA

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WITH THE TECHNICAL ASSISTANCE OF JAUNEITA OGG

The concentration and total amount of one or both catecholamines, epinephrine, and norepinephrine, is usually greatly increased in pheochromocytomas over that in the adrenal gland. Their concentration in urine and plasma of patients with pheochromocytoma is increased also, either continuously or intermittently, because of release from the tumors. Quantitative and qualitative tests for urinary catecholamines based on oxidation to fluorescent products by alkaline ferricyanide were compared. The relative simplicity of the Hingerty qualitative test for free catecholamines was found to be useful as a screening test for 24 hour specimens of urine when the tumor was actively secreting catecholamines. The quantitative test as used by Sobel and Henry provided further information on the total amount of catecholamines in hydrolyzed urine. Rigid exclusion of medication with salicylates and other drugs that also produce fluorescence was essential for all tests. Measurements of the concentration of plasma catecholamines by the method of Weil-Malherbe and Bone were useful, even when the urine tests gave positive results. When the tumor was very small or was not actively secreting catecholamines, the only positive chemical test for pheochromocytoma was obtained in blood drawn during a spontaneous attack of paroxysmal hypertension, or during an attack provoked by injection of histamine.

STUDIES of catecholamines in the adrenal medulla, which began over a century ago with the observation of Vulpian[1] of a rose-red color in extracts of this tissue, led to the isolation of crystalline epinephrine from the adrenal in 1901 by Takamine.[2] Evidence gradually accumulated that epinephrine was stored in large amounts in pheochromocytomas. Rabin,[3] in 1929, examined a pheochromocytoma found at necropsy by the method of Folin, Cannon, and Dennis and found much more epinephrine than is found in normal adrenal glands. Crystalline epinephrine was isolated from such a tumor for the first time in 1936 by Kendall.[4] Crystalline epinephrine was subsequently isolated from a series of these tumors in his laboratory in quantities per gram (Table I) which equaled or exceeded those which were isolated as a by-product from the beef adrenal glands[5] which were extracted primarily for cortical steroids.

By 1949, it was apparent that small amounts of norepinephrine were present in the adrenal gland in addition to the larger amounts of epinephrine.[6, 7] Bergström and associates[8] isolated norepinephrine from the adrenal medulla.

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Tullar[9] isolated norepinephrine from U. S. P. epinephrine and showed that norepinephrine made up 10 to 25 per cent of the mixed crystals. In 1949, Holton[10] and Goldenberg and co-workers[11] demonstrated larger amounts of norepinephrine in pheochromocytomas than in the adrenal gland. In 1950, we examined by paper chromatography the crystalline material which Kendall had isolated earlier from three pheochromocytomas and found that 10, 20, and 80 per cent of the catecholamines, respectively, were norepinephrine.

The relative proportion of the two principal catecholamines in pheochromocytomas varies greatly as does the total amount of the catecholamines, but in many of the pheochromocytoms, norepinephrine is more abundant than epinephrine.[11, 12] Recently the precursor of these catecholamines, 3-hydroxytyramine, was found in a few of these tumors[13-15] and dihydroxyphenylalanine in one.[13] Small amounts of the metabolite, methoxynorepinephrine, also have been found.[16]

The continuous or intermittent release of epinephrine and norepinephrine from these tumors produces sustained or paroxysmal hypertension. Detection of increased amounts of these catecholamines in blood is helpful in establishing the diagnosis of hypertension due to a pheochromocytoma.[17] Manger and coworkers[15] measured the concentration of catecholamines in the blood of 13 patients who had positive pharmacologic tests for pheochromocytoma and were subsequently proved to have pheochromocytomas. High values of epinephrine-like substances from 4.2 to 17.7 μg per liter of plasma were found in 9 patients who had sustained hypertension, and in 2 of the 4 patients with paroxysmal hypertension. The average concentration in 11 normal subjects was 2.4 μg, and in 25 patients without pheochromocytomas, the concentrations were not greater than 3.1 μg per liter of plasma.

Increased amounts of catecholamines are excreted in the urine by patients with pheochromocytomas.[18] Even larger amounts of the metabolites 3-methoxy-4-hydroxymandelic acid[19] and the methoxy derivatives of epinephrine and norepinephrine[20] have been found in the urine. Studies of the urine, therefore, are useful in making the diagnosis.

We wish to report our experience with the measurement of catecholamines in blood and urine as tests for pheochromocytoma. We also have measured the catecholamines in 54 of the 56 pheochromocytomas from 48 patients.

METHODS

Plasma Pressor Amines.—The pressor amines, or catecholamines, were determined by the method of Weil-Malherbe and Bone.[21] The specimens of blood were promptly mixed with a sodium fluoride–sodium thiosulfate solution and centrifuged. The pressor amines of the plasma were adsorbed on an alumina (Merck's or Alcoa) column, eluted, and condensed with ethylenediamine. The fluorescence was measured in a modified Pfaltz-Bauer fluorometer and compared with that of an epinephrine standard which had been added to plasma from a normal dog and run through a column before condensation with ethylenediamine. At intervals during this study, the fluorescence of epinephrine which was added to blood and carried through the entire procedure was compared with the fluorescence of epinephrine added to acetic acid just before the reaction with ethylenediamine. The mean recovery in 38 tests was 99.5 per cent with a standard deviation of 11.0. The concentration of catecholamines was expressed in micrograms of epinephrine-like substance per liter of

plasma.[22] At the wavelengths used for activation (436 mμ) and measurement of fluorescence (578 mμ), norepinephrine contributed only 30 per cent as much fluorescence as did epinephrine.

Corning filters 3389 and 5113 were used for the incident light and filters 3480 and 4303 for the fluorescent light.

Measurements of the individual catecholamines by the chemical method[22] requires additional time and larger samples of blood. Such measurements are not essential for the diagnosis of pheochromocytoma and have been omitted.

Urinary Catecholamines.—

Quantitative tests: Twenty-four hour specimens of urine were collected in bottles which contained 15 ml. of 6N acetic acid. The catecholamines were determined by the method of von Euler and Floding,[23] as used by Sobel and Henry.[24] Aliquots of urine, of 20 ml. each, were hydrolyzed with H_2SO_4 at a pH of less than 2, and the catecholamines were adsorbed on alumina which had been washed with acid and dried at 300° C. as described by Weil-Malherbe and Bone.[21]

The catecholamines were eluted with 0.2N acetic acid and oxidized with potassium ferricyanide at pH 6.5. At this pH equal amounts of epinephrine and norepinephrine produced approximately equal amounts of fluorescence. This was measured in the Aminco-Bowman spectrophotofluorometer. The ingestion of many drugs may interfere with this method if they or their products produce fluorescence under the conditions used. Crout and Sjoerdsma[25] found that after ingestion of three bananas, which contain norepinephrine,[26] the urine contains hydrolyzable conjugates as great as may be found in the presence of a pheochromocytoma. The quantitative test for total catecholamines then became unreliable, whereas the qualitative test for free catecholamines was still useful.

Qualitative tests: Ten per cent of the 24 hour output of urine was used without acid hydrolysis in the qualitative test. The catecholamines were adsorbed on alumina, eluted, and oxidized with potassium ferricyanide as described by Hingerty.[27] For a standard, norepinephrine was added to a similar volume of normal urine, and this was treated in a similar manner. The fluorescence of each unknown was compared visually with that of the standard under an ultraviolet lamp. Specimens of urine which developed more fluorescence than the standard were considered positive for catecholamines and were used for further quantitative studies. This qualitative test cannot be used if the patient has ingested aspirin or other salicylates, since these substances produce a blue fluorescence which completely obscures the green fluorescence of the catecholamines. The ferric chloride test for salicylates is useful for excluding urines which contain salicylates from the qualitative tests.

Metabolites of Catecholamines in Urine.—We measured the 3-methoxy-4-hydroxymandelic acid in the urine by the paper chromatographic technique of Armstrong and associates.[19, 28] Aliquots of urine were acidified with 3N HCl to a pH of 0.5 to 1.5. Aromatic acids were extracted into 2 volumes and then in 1 volume of ethyl acetate. The extract was dried in air and dissolved in a small amount of absolute alcohol. Aliquots of the extract corresponding to 1.0 mg. or 0.1 mg. creatinine were applied to 12 by 12 inch sheets of Whatman No. 1 filter paper with standard amounts (0.5, 1.0, 1.5, and 2.0 μg) of 3-methoxy-4-hydroxymandelic acid at 1 inch intervals beginning 4 inches above the unknown. The sheets were developed in isopropyl alcohol—NH_4OH—water (80:2:18), and benzene-propionic acid (100:70:5) and sprayed with diazotized *p*-nitroaniline. Estimations were made visually by comparison with the standard spots.

The methoxy derivatives of epinephrine or norepinephrine in the urine were measured by the method of Axelrod and associates.[29] The urine was incubated with beta-glucuronidase; the methoxy compounds were extracted into ethylene dichloride which contained 2 per cent isoamyl alcohol and then from the organic phase into 0.1N HCl. The fluorescence of the combined metanephrine and normetanephrine was measured in an Aminco-Bowman spectrophotofluorometer.

Catecholamines in Pheochromocytomas.—

Paper chromatography: The tumors, which had been frozen in powdered dry ice immediately after removal, were crushed between chilled steel blocks. Samples, usually weighing approximately 7 grams each, were extracted overnight in 5 volumes of 95 per cent ethanol which contained 100 mg. of sodium bisulfite and 0.1 ml. of glacial acetic acid, as recommended by E. C. Kendall. The extracts were filtered with suction and the residue was washed with ethanol. Aliquots of the extracts were dried *in vacuo*, dissolved in acid alcohol (1 part of concentrated HCl diluted to 100 ml. with ethyl alcohol), and pipetted on strips or sheets of Whatman No. 4 or 3-MM. filter paper. The chromatograms were developed, usually overnight, either by ascent or descent in 75 per cent phenol in a jar which contained a beaker of 15 per cent sodium bisulfite, or in a 4:1:5 mixture of butanol–acetic acid–water. After drying, the papers were sprayed with the potassium ferricyanide reagent of James.[30] Comparison of the size and density of the pink color in the unknowns with those of standard amounts of epinephrine and norepinephrine run on the same sheet made it possible to estimate the amount of these catecholamines in the tumors. Observation of the fluorescent products, produced either by spraying the sheets with ferricyanide or ethylenediamine and ammonia[31] in ultraviolet light, was particularly helpful in the detection of 3-hydroxytyramine in the tumors.

The methoxy compounds in pheochromocytomas were extracted and separated on paper chromatograms by the method of Sjoerdsma and co-workers,[16] and the blue bands which appeared when the strips were sprayed with N,2,6-trichloro-*p*-quinoneimine and borate buffer were compared with standards run on parallel strips. In other experiments, aliquots of the alcoholic extracts used for catecholamines were taken to dryness *in vacuo* at 37° C., then dissolved in the borate buffer, extracted with butanol and, after the addition of heptane, extracted into 0.5N HCl as in Sjoerdsma's method. The methoxy compounds were separated on paper chromatograms usually in isopropanol and 5 per cent NH_3 (8:2), and eluted with 0.1N HCl after location by comparison with guide strips which were sprayed with Armstrong's diazotized *p*-nitroaniline reagent. This reagent was then added to the eluted methoxy compounds, and the optical density of each was measured in a Beckman spectrophotometer at 510 mμ.

Chemical techniques: The method of Weil-Malherbe and Bone[21] for measurement of catecholamines was used for some of the pheochromocytomas as in the earlier series of Manger and co-workers.[15] The fluorescence of total epinephrine-like substances was measured in the modified Pfaltz-Bauer fluorometer, and of the individual catecholamines in the Aminco-Bowman spectrophotofluorometer. More recently, the tumors have been analyzed by the method of Sobel and Henry.[24]

RESULTS

Pheochromocytomas.—The tumors varied greatly in size, in total content of catecholamines, and in the relative percentage of epinephrine and norepinephrine. The concentration of both catecholamines together varied from 0.3 to 17.5 mg. per gram of tumor in patients with paroxysmal hypertension, and norepinephrine accounted for 50 per cent or more of the catecholamines in 16 of the 32 tumors from these 29 patients (Tables I to III).

In the tumors from patients with sustained hypertension, the concentration of catecholamines varied from 0.5 to 8.4 mg. per gram of tumor. There was one exception in Patient 39 with metastatic lesions which occurred 2 years after the first pheochromocytoma was removed and which contained no catecholamines after surgical removal. Fifty per cent or more of the catecholamines was norepinephrine in 20 of the 22 tumors so studied. All but 5 of the total of 54 tumors which were analyzed were found within the adrenal. The weight of the

tumors varied from 1.2 to 700 grams, and the total amount of catecholamines found varied from 8.9 to 5,200 mg. except for the metastatic lesion just described. The different tumors contained varying amounts of stroma with necrosis and were not always uniform throughout the entire structure. The concentrations shown, therefore, represent average values for the total tumors. Representative samples of the larger tumors and the major portion of the smaller tumors were analyzed.

TABLE I. CATECHOLAMINES IN TUMORS OF PATIENTS WITH PHEOCHROMOCYTOMAS: EARLY STUDIES

PATIENT AND TYPE OF HYPERTENSION*	AGE (YR.)	SEX	TUMOR		
				CATECHOLAMINES	
			WEIGHT (GM.)	MG./GM.†	NOREPINEPHRINE (% OF TOTAL)
1 P	41	F	198	4.1‡	12
2 P	59	M	70	1.5‡	20
3 S	21	F	50	1.3‡	80
4 S	18	M	250	3.8	67
S	21	M	23	2.5	100
5 P	52	F	42	6.2	20
6 S	48	F	36	6.9	72
7 S	25	F	15	2.1	95
8 S	47	M	350	6.0	50
9 P	36	F	7	14.0	50
10 P	38	F	50	3.3	33
11 S	18	F	36	6.0	50
			73	8.0	50
12 S	28	F	323	6.0	83
			105	7.0	71
13 P	26	M	72	15.0	66
			28	15.0	66
			5	17.5	43
14 P	38	M	15	3.1	22
15 P	46	F	575	9.0	89
16 P	39	M	53	8.5	18
17 S	25	F	13	3.5	100
			7	3.5	100
18 P	55	F	250	1.4	89
19 S	26	M	70	0.5	95
20 P	39	M	120	6.2	20
21 S	43	F	165	0.8	100
22 P	49	F	45	7.5	50
23 P	53	F	14	3.1	97
24 P	34	M	105	0.3	100
25 S	49	M	30	1.4	89
26 P	52	M	105	8.4†	11

*In this and the following tables P = paroxysmal hypertension and S = sustained hypertension.

†The concentration of catecholamine was determined by paper chromatography in all but one case (Patient 26). In that case the method for total epinephrine-like substances of Weil-Malherbe and Bone was used.

‡Crystalline "epinephrine" previously isolated by Kendall was examined.

The concentrations of catecholamines found in the pheochromocytomas were generally greater than those found in grossly normal adrenal glands which were removed from seven patients because of inoperable carcinoma in five, Cushing's disease in one, and Simmonds' disease in another. Concentration of catecholamines in the glands measured by paper chromatography varied

from 0.10 to 0.75 mg. per gram, and the weight of the individual glands varied from 4.2 to 9.2 grams. The total amount of catecholamines varied from 0.8 to 4.4 mg. per adrenal and most of this was epinephrine. In one patient 12 per cent of the catecholamines was norepinephrine, in another 2 per cent, whereas none was found in the others, in the amounts which we used for the paper chromatograms. It is possible that the presence of systemic disease and the necessary surgical procedures may have reduced the amount of catecholamines in these glands. Since cortical tissue was included, it is probable that the concentration of catecholamines in the normal adrenal medulla may be as great as in some pheochromocytomas.

3-Hydroxytyramine was found in relatively small amounts in four pheochromocytomas. The amounts were estimated in three of these tumors by paper chromatography to be 0.1, 0.1 and 0.3 mg. per gram. 3-Methoxynorepinephrine was found in five and 3-methoxyepinephrine in six of the pheochromocytomas that have been studied recently. The amounts were very small in all cases, being less than the 25 μg per gram which Sjoerdsma and co-workers[16] had found of methoxyepinephrine in such a tumor.

Catecholamines in Plasma.—The concentration of catecholamines, or pressor amines, in the plasma has been measured in 1,127 patients whose symptoms suggested the possible presence of a pheochromocytoma, but who were subsequently considered to be free of this tumor. The mean concentration of epinephrine-like substances was 2.4 μg per liter, with a standard deviation of $\pm$1.1. The cumulative frequency distribution curve of the concentration in plasma showed that 95 per cent of the values were less than 4.5 μg per liter (Fig. 1). Seven additional specimens with abnormally high values from 10.2 to 35.0 μg per liter were considered spurious and were excluded from this series of essentially normal values. Information sometimes was obtained concerning the ingestion of drugs which produced fluorescence in the sample analyzed. A few of these patients were jaundiced or had some evidence of renal insufficiency, which, according to Zileli and co-workers,[32] may produce an elevation in the plasma catecholamines.

The concentration of catecholamines was measured in the plasma of 18 patients with pheochromocytomas which were subsequently removed surgically. Concentrations of 6.0 to 86.5 μg per liter of plasma, which were significantly greater than those in normal subjects, were found in 15 of these patients before histamine or Regitine was given. Tests run on different days showed high but variable values. Also the response to the histamine test[33] varied greatly because of previous medication. In some patients with high blood levels, no further increase was produced by histamine.[34] In some, dramatic increases were produced, the greatest being from 86.5 to 360 μg per liter (Patient 30, Table II). Two days later when the histamine test was repeated in this patient, a smaller increase was found, namely from 47.0 to 58.0 μg. Normal levels of plasma catecholamines are usually found in patients with paroxysmal hypertension during normotensive phases. Two patients with

TABLE II. CATECHOLAMINES IN TUMORS AND BLOOD OF PATIENTS WITH PHEOCHROMOCYTOMAS

PATIENT AND TYPE OF HYPERTENSION	AGE (YR.)	SEX	DRUG GIVEN	BLOOD PRESSURE BEFORE OPERATION (MM. HG)		PLASMA CATECHOLAMINES* (μG/L.)				TUMOR		
						BEFORE OPERATION		AFTER OPERATION			CATECHOLAMINES	
				BEFORE DRUG	AFTER DRUG	BEFORE DRUG	AFTER DRUG	BEFORE DRUG	AFTER DRUG	WEIGHT (GM.)	MG./GM.	NOR-EPINEPHRINE (% OF TOTAL)
18 P	58	F	Histamine	126/60	244/160		17.0			Nodule	1.7	98
27 P	67	F	Histamine	130/80	190/136	21.3	24.3			140	2.2†	50
28 P	29	F	Histamine	112/82	174/120	8.2	14.5			192	7.0	28
29 P	50	F	Histamine	126/78	162/96‡	6.8§	6.4§			72	3.0†	100
						6.0	6.2	3.0				
30 P	44	F	Histamine	166/110	250/172	86.5	360.0	2.4	5.4	700	2.2‖	7‖
						47.0	58.0					
31 P	32	M	Histamine	140/110	290/190	4.7	299.9			50	1.1	75
32 P	38	F	Histamine	110/66	184/90	5.5	7.1			350	3.9	97
33 P	50	F	Histamine	160/114	264/164	4.5	18.1			190	1.2	33
34 P	65	M	Histamine	174/106	274/180		36.2			80	8.3	60
35 S	32	F	Histamine	172/130	274/190	24.9	27.9	11.0	11.4	37		
			Regitine			40.0	39.3	12.2	11.3			
36 S	57	F	Regitine	214/126	138/90	12.7	13.6			60	8.4	71
37 S	52	M	Regitine	194/124	150/94	14.4	19.9			62	4.5	56
38 S	18	F	Regitine	180/140	80/46	18.1	16.5	3.0	3.3	45		
39 S¶	45	F	Regitine	264/150	138/100	18.8	19.5			110	0.8	94‖
			Histamine					3.5	4.0			
40 S	39	M	Regitine	180/130**	104/60**	17.1	30.8**			80	3.2†	50

*The concentrations of catecholamines in the plasma before and after injection of histamine or Regitine.
†Determined by the method of Weil-Malherbe and Bone.
‡Same as on cold pressor test.
§Receiving antihypertensive drugs.
‖Determined by the method of Sobel and Henry.
¶In Patient 39, 0.1 mg. per gram of 3-hydroxytyramine was found in the tumor by paper chromatography.
**Spontaneous attack when patient turned on side; Regitine then given.

TABLE III. CATECHOLAMINES IN TUMORS, BLOOD, AND URINE OF PATIENTS WITH PHEOCHROMOCYTOMAS

PATIENT AND TYPE OF HYPERTENSION	AGE (YR.)	SEX	DRUG GIVEN	BLOOD PRESSURE BEFORE OPERATION (MM. HG)		PLASMA CATECHOLAMINES* (μG/L.)				URINARY CATECHOLAMINES			TUMOR		
						BEFORE OPERATION		AFTER OPERATION		BEFORE OPERATION		AFTER OPERATION		CATECHOLAMINES	
				BEFORE DRUG	AFTER DRUG	BEFORE DRUG	AFTER DRUG	BEFORE DRUG	AFTER DRUG	μG/24 HR.	NOREPINEPHRINE (%)	μG/24 HR.	WEIGHT (GM.)	MG./GM.	NOREPINEPHRINE (% OF TOTAL)
41† P	32	F	Histamine	172/130	274/190	12.8	79.3	1.1	1.6	1,260	25	37	100	13.6	35
42† P	60	F	Histamine	166/96	250/150		11.9	0.9	1.3						
			Regitine				10.4			560			108	15.6	31
43† P	47	M	Histamine	124/88	194/134	2.1	9.6			64			1.2	7.4	100
44 P	16	F	Histamine	146/114	214/154	6.6	78.1		2.1	890	70	82	30	2.1	95
										1,000					
										1,260					
45 P	50	M	Histamine	150/114	270/230	2.2	21.6			317	0		30	8.3	45
46 S	44	F	Regitine	244/120	114/48	9.2	10.6			1,080		280	40	6.5	90
			Histamine					1.7		2,780		340			
										1,250	52				
47 S	41	F	Regitine	184/134	128/95	9.5	10.7								
			Histamine						2.4	2,750	74	240	270	9.8	39
48 P	46	M	Histamine		Negative test	3.1	3.0			816		257	32	14.2	15
			Hyperventilation	194/120			3.9‡								
							4.4‡								
							9.8‡								
39§ S	47	F	Regitine	194/114	126/74	6.0				978	86	690	65	0	
			Histamine			2.0			3.7	1,294	92				

*The concentrations of catecholamines in the plasma before and after injection of histamine or Regitine.

†The 3-hydroxytyramine in the tumor was determined by paper chromatography. In Patients 42 and 43 estimates of 0.1 and 0.3 mg. per gram were made.

‡These concentrations of plasma catecholamines were found during a period of strenuous hyperventilation which followed the negative histamine test and led to a spontaneous attack with plasma concentrations of 9.8 μg per liter. One month previously, negative histamine tests were found with levels of 2.1 to 1.2 and 3.6 to 3.8 μg before and after the histamine injection on two occasions. Two years previously, concentrations before and after the injection of histamine were 4.2 to 5.2, 3.2 to 3.7, and 3.0 to 5.5 μg per liter on three occasions.

§Metastatic tumor.

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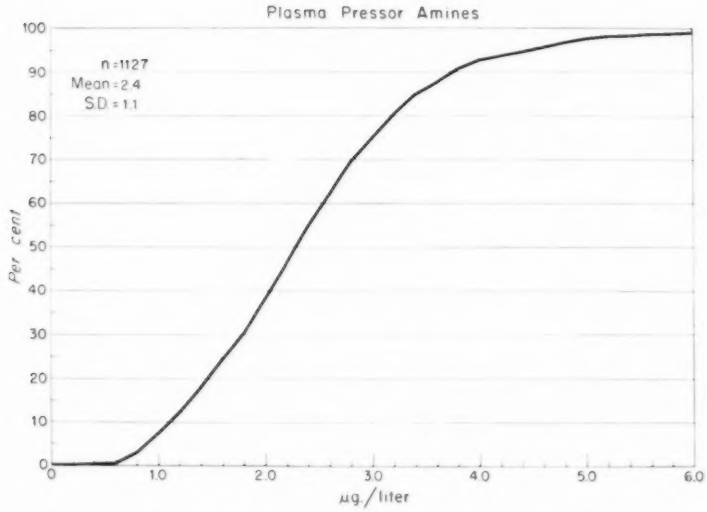

Fig. 1.—Cumulative frequency distribution curve of the concentration of plasma pressor amines, or catecholamines, in patients without pheochromocytomas. The values given are in micrograms of epinephrine-like substance per liter of plasma.

normal levels of 2.1 and 2.2 μg per liter showed increases to 9.6 and 21.6 μg after the injection of histamine (Patients 43 and 45, Table III). One patient (Patient 48) with normal levels of 3.1 and 3.0 μg per liter before and after histamine was asked to hyperventilate strenuously and then had a spontaneous attack of hypertension with a blood level of 9.8 μg per liter. In a series of 48 patients without pheochromocytoma, the mean concentration of epinephrine-like substances in plasma was 2.0 μg per liter ±0.8 (S. D.) before and 2.1 μg ±0.7 after the histamine test.

The concentration of catecholamines in plasma was determined after operation in 11 of the 23 patients. Normal values were found in 10 patients and elevated values indicative of residual functioning tissue were found in one patient (Patient 35, Table II).

Urinary Catecholamines.—

Quantitative tests: The mean 24 hour output of catecholamines in 647 specimens of urine from individuals, subsequently considered free of pheochromocytomas, was 119 μg with a standard deviation of ±73 and a range from 10 to 643. The cumulative frequency distribution curve showed that 95 per cent of the values were less than 250 μg (Fig. 2), and these values were considered normal. Some of the patients with abnormal values subsequently were found to have been ingesting drugs which may have been responsible for some of the fluorescence in the urine since omission of the drugs was followed by the appearance of normal concentrations of catecholamines in the urine.

The catecholamines in the urine of 9 patients, later proved to have pheochromocytomas, varied from 317 to 2,780 μg in 24 hours with one exception (Table III). The normal excretion of 64 μg in 24 hours was found in the

patient with the 1.2 gram tumor. Normal values were generally found for urinary catecholamines in patients after surgical removal of the tumor. The high value found in Patient 39 (Table III) indicates that probably additional functioning tissue is present.

Qualitative tests: The qualitative test for free catecholamines in the urine gave positive results on 9 patients whose quantitative test gave positive results. The qualitative test was used on 629 of the 646 specimens of urine from patients without proved pheochromocytomas. In 80 per cent of these specimens, the fluorescence was found to be less than in the standard, and thus the 24 hour output of catecholamines expressed as norepinephrine was less than 180 μg. Results of these tests were considered negative. In 20 per cent of the specimens, the tests gave indeterminate results since the fluorescence was blue instead of the yellow-green color produced by catecholamines. Subsequently, when a positive result in one patient was followed by indeterminate results, Dr. F. T. Maher noted that the patient had ingested Empirin and that a test of the urine was positive for salicylates. This and many additional observations have shown that the blue fluorescence was associated with the ingestion of salicylates. Omission of such medicaments removed this complication. The qualitative test never gave a positive result when the quantitative test gave a negative result. Nor did it give a negative test in any of the cases of pheochromocytoma in which a quantitative test gave a positive result. Results of the qualitative test were negative in several instances in which results of the quantitative test were positive because of ingestion of drugs. The qualitative test is now being used routinely as a test for pheochromocytoma.

3-Methoxy-4-Hydroxymandelic Acid and Methoxy Compounds.—3-Methoxy-4-hydroxymandelic acid has been shown by Armstrong and co-workers[19] to be

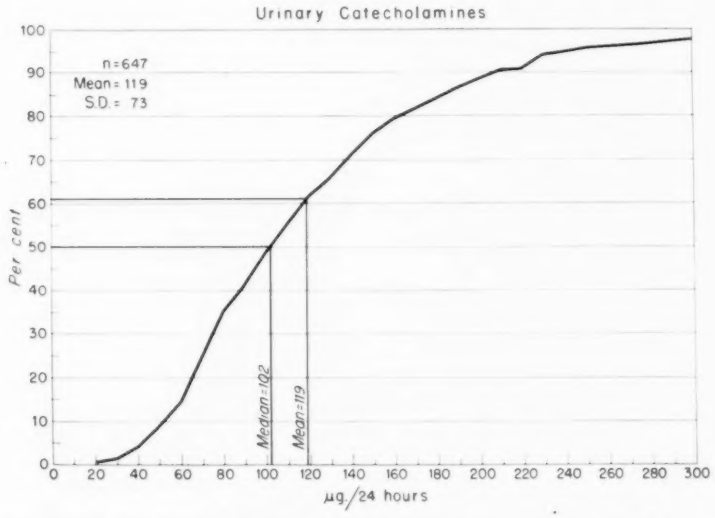

FIG. 2.—Cumulative frequency distribution curve of the urinary output of catecholamines in 24 hours in patients without pheochromocytomas.

an important metabolite of catecholamines in the urine. They have found concentrations of 1.5 to 3.0 μg of 3-methoxy-4-hydroxymandelic acid per milligram of creatinine in most normal individuals, and from 9 to 90 μg in patients in whom pheochromocytomas were found later at operation. Using their technique, we have found concentrations of 5.0 to 62 μg per milligram of creatinine in 6 patients with proved pheochromocytomas (Table IV) in contrast to values of 4.0 μg or less in 15 of 17 patients without proved pheochromocytomas. Values of 10.0 and 15.0 μg were found, however, in the other two patients, although they had normal concentrations of catecholamines, namely 154 and 49 μg in 24 hours. Gitlow and associates[35] have found no overlapping in values for 3-methoxy-4-hydroxymandelic acid between normal subjects and patients with pheochromocytomas when they ingested no fruit, coffee, tea, or substances which contained appreciable quantities of vanilla for a period of 24 hours before urine was collected.

TABLE IV. URINARY METABOLITES OF CATECHOLAMINES

PATIENT	3-METHOXY-4-HYDROXYMANDELIC ACID		METHOXY-CATECHOLAMINES
	μG/MG. OF CREATININE	MG. IN 24 HR.	(MG. IN 24 HR.)
39	7.5	6.3	
	17.5	12.1	
42	15.0	9.3	
44	25.0	28.3	6.6
	25.0	23.9	5.7
	50.0	53.8	5.8
45	5.0	3.1	6.6
46	10.0	4.4	
	10.0	9.6	
	15.0	11.4	
47	62.0	85.7	21.0

Methoxy derivatives of the catecholamines were measured in the urine of three patients with pheochromocytomas. Urinary excretions of 6.6, 5.7, and 5.8 mg. in 24 hours were found in one of these patients, and 21.0 and 6.6 mg. in two other patients (Table IV). Three patients without proved pheochromocytomas excreted 4.1, 5.8, and 7.3 mg. in 24 hours.

COMMENT

Increases in the catecholamine content of blood associated with the presence of pheochromocytoma were readily detected by the method of Weil-Malherbe and Bone. The rate of release of catecholamines from these tumors varied considerably in different patients. In some patients with pheochromocytomas, the concentration of catecholamines in the blood was elevated continuously, in others only occasionally during spontaneous attacks. In the latter group, the injection of histamine usually would release catecholamines from the tumor in quantities sufficient to give a marked elevation in both the blood pressure and the level of blood catecholamines within 2 to 4 minutes after the injection. Care must be taken to eliminate medication with drugs capable of producing an interfering fluorescence prior to using this blood test.[36]

The von Euler and Floding method for the quantitative determination of catecholamines in urine as modified by Sobel and Henry also is useful for the detection of pheochromocytoma. Even greater care must be taken to exclude medication with other substances which produce fluorescent products in the urine. Also, if the tumor is very small or if the tumor is not releasing catecholamines in significant amounts during the test period, a negative result may be obtained even when a tumor is present.

The Hingerty[27] qualitative test for catecholamines in the urine is a useful and simple test for pheochromocytoma. Medication with salicylates should be avoided before this test. This precaution should also be taken prior to estimation of the metabolite, 3-methoxy-4-hydroxymandelic acid by paper chromatography, according to Armstrong.[28]

The variations in concentration of catecholamines in the 54 pheochromocytomas examined, in the total quantity of these amines stored in each tumor, and in the relative amounts of epinephrine and norepinephrine in each are in general similar to those reported for other pheochromocytomas by many investigators.[7, 15, 37] The occurrence of significant amounts of 3-hydroxytyramine in a few of these tumors in addition to other catecholamines was not associated with any differences in the symptoms presented by the patients. The quantities of metanephrine and normetanephrine which we have found very recently are of the same order of magnitude as the quantity of normetanephrine found by Sjoerdsma and co-workers[37] in a pheochromocytoma. These methoxy metabolites exist in the tumors in almost trace amounts compared to those of the catecholamines.

Normetanephrine[20] and 3-methoxy-4-hydroxymandelic acid[19] are major metabolites in the urine of patients with pheochromocytomas, and they occur in much larger amounts than do the catecholamines. The O-methylated compounds of urine account for about 55 per cent of the metabolic products of norepinephrine in the normal human subject according to Goodall and associates.[38] The total quantity of these excretory products was higher in some of our patients with pheochromocytoma than in normal subjects.

Blaschko and associates[39] have shown that catecholamines are stored in cytoplasmic granules in the adrenal medulla in high concentration. They are in close association with adenosine triphosphate which Falck and associates[40] have found also occurs in high concentration in these medullary granules. Little is known concerning the mechanism of release of the catecholamines from the granules or cells of either the normal adrenal medulla or pheochromocytoma. The amount of catecholamines in the tumors does not appear to be directly proportional to the concentration of catecholamines in the plasma or urine or to the concentration of their metabolites in the urine. It is obvious that the rate of turnover of the catecholamines in the large tumors must be less than in the three patients studied by Sjoerdsma and co-workers,[37] who had a turnover of norepinephrine with a half-life of about 10 hours.

SUMMARY

The qualitative test of Hingerty for free urinary catecholamines in 24 hour specimens of urine provides a useful procedure for the detection of pheochromocytomas which are actively releasing catecholamines. When results are positive, quantitative determination of the catecholamines in hydrolyzed urine provides additional useful information, as does the measurement of the catecholamines in plasma, in establishing the diagnosis.

When pheochromocytomas are not releasing catecholamines in quantities sufficient to cause an elevation in blood pressure, they are not detectable by these chemical procedures. The injection of histamine under properly controlled conditions or a spontaneous attack, however, will usually release catecholamines from these tumors in sufficient amounts to produce an immediate rise in the concentration of catecholamines in the blood. The amount of catecholamines in the blood and urine is not an accurate index to the very large amounts of either epinephrine or norepinephrine, or both, often stored in pheochromocytomas.

We are grateful to the Winthrop Laboratories for gifts of crystalline L-arterenol bitartrate and L-epinephrine bitartrate, to M. D. Armstrong of the Fels Research Institute at Yellow Springs, Ohio, for 3-methoxy-4-hydroxymandelic acid and a copy of his detailed procedure for the determination of this substance by paper chromatography,[28] to Bernhard Witkop of the National Institute of Arthritis and Metabolic Diseases, for metamethoxytyramine, 1-metamethoxy-norepinephrine, and 1-meta-O-methylepinephrine, and Robert Gage for assistance in the statistical analysis of our data.

REFERENCES

1. Vulpian, M.: Note sur quelques reactions propres à la substance des capsules surrénales, Compt. rend. Acad. d. sc., Par. **43**: 663, 1856.
2. Takamine, J.: Adrenalin, the Active Principle of the Suprarenal Glands and Its Mode of Preparation, Am. J. Pharmacol. **73**: 523, 1901.
3. Rabin, C. B.: Chromaffin Cell Tumor of the Suprarenal Medulla (Pheochromocytoma), Arch. Path. **7**: 228, 1929.
4. Kelly, H. M., Piper, M. C., Wilder, R. M., and Walters, W.: Case of Paroxysmal Hypertension With Paraganglioma of the Right Suprarenal Gland, Proc. Staff Meet. Mayo Clin. **11**: 65, 1936.
5. Kendall, E. C.: A Chemical Study of the Active Constituents of the Suprarenal Gland, J. Biol. Chem. **92**: lvi, 1931.
6. Holtz, P., and Schümann, H. J.: Arterenol Content of the Mammalian and Human Adrenal Medulla, Nature, London **165**: 683, 1950.
7. Von Euler, U. S., and Hamberg, U.: 1-Noradrenaline in the Suprarenal Medulla, Nature, London **163**: 642, 1949.
8. Bergström, S., von Euler, U. S., and Hamberg, U.: Isolation of Norądrenaline From Suprarenal Medulla, Acta physiol. scandinav. **20**: 101, 1950.
9. Tullar, B. F.: The Separation of L-Arterenol From Natural U. S. P. Epinephrine, Science **109**: 536, 1949.
10. Holton, P.: Noradrenaline in Tumors of the Adrenal Medulla, J. Physiol. **108**: 525, 1949.
11. Goldenberg, M., Faber, M., Alston, E. J., and Chargaff, E. C.: Evidence for the Occurrence of Norepinephrine in the Adrenal Medulla, Science **109**: 534, 1949.
12. Von Euler, U. S., and Ström, G.: Present Status of Diagnosis and Treatment of Pheochromocytoma, Circulation **15**: 5, 1957.
13. Weil-Malherbe, H.: Phaeochromocytoma: Catechols in Urine and Tumour Tissue, Lancet **2**: 282, 1956.
14. McMillan, M.: Identification of Hydroxytyramine in a Chromaffin Tumour, Lancet **2**: 284, 1956.

15. Manger, W. M., Flock, E. V., Berkson, J., Bollman, J. L., Roth, G. M., Baldes, E. J., and Jacobs, M.: Chemical Quantitation of Epinephrine and Norepinephrine in Thirteen Patients With Pheochromocytoma, Circulation **10**: 641, 1954.
16. Sjoerdsma, A., King, W. M., Leeper, L. C., and Udenfriend, S.: Demonstration of the 3-Methoxy Analog of the Norepinephrine in Man, Science **127**: 876, 1958.
17. Von Euler, U. S.: Catechol Hormones in Blood, Ciba Foundation Colloquia on Endocrinology **11**: 379, 1957.
18. Engel, A., and von Euler, U. S.: Diagnostic Value of Increased Urinary Output of Noradrenaline and Adrenaline in Phaeochromocytoma, Lancet **2**: 387, 1950.
19. Armstrong, M. D., McMillan, A., and Shaw, K. N. F.: 3-Methoxy-4-hydroxy-D-mandelic Acid, a Urinary Metabolite of Norepinephrine, Biochim. et biophys. acta **25**: 422, 1957.
20. LaBrosse, E. H., Axelrod, J., and Sjoerdsma, A.: Urinary Excretion of Normetanephrine by Man, Fed. Proc. **17**: 386, 1958. (Abst.)
21. Weil-Malherbe, H., and Bone, A. D.: The Chemical Estimation of Adrenaline-Like Substances in Blood, Biochem. J. **51**: 311, 1952.
22. Manger, W. M., Baldes, E. J., Flock, E. V., Bollman, J. L., Berkson, J., and Jacobs, M.: A Method for Quantitative Estimation of Epinephrine and Norepinephrine: Preliminary Report, Proc. Staff Meet. Mayo Clin. **28**: 526, 1953.
23. Von Euler, U. S., and Floding, I.: A Fluorimetric Micromethod for Differential Estimation of Adrenaline and Noradrenaline, Acta physiol. scandinav. **33** (suppl. 118): 45, 1955.
24. Sobel, C., and Henry, R. J.: Determination of Catecholamines (Adrenalin and Noradrenalin) in Urine and Tissue, Am. J. Clin. Path. **27**: 240, 1957.
25. Crout, J. R., and Sjoerdsma, A.: Catechol Amine Excretion After Banana Feeding (Letter to the Editor), J. Pharm. & Pharmacol. **11**: 190, 1959.
26. Waalkes, T. P., Sjoerdsma, A., Creveling, C. R., Weissbach, H., and Udenfriend, S.: Serotonin, Norepinephrine, and Related Compounds in Bananas, Science **127**: 648, 1958.
27. Hingerty, D.: Thirty-Minute Screening Test for Phaeochromocytoma, Lancet **1**: 766, 1957.
28. Armstrong, M. D.: Personal communication.
29. Axelrod, J., Inscoe, J. K., Senoh, S., and Witkop, B.: O-Methylation, the Principal Pathway for the Metabolism of Epinephrine and Norepinephrine in the Rat, Biochim. et biophys. acta **27**: 210, 1958.
30. James, W. O.: Demonstration and Separation of Noradrenaline, Adrenaline and Methyladrenaline, Nature, London **161**: 851, 1948.
31. Weil-Malherbe, H., and Bone, A. D.: The Estimation of Catecholamines in Urine by a Chemical Method, J. Clin. Path. **10**: 138, 1957.
32. Zileli, M. S., Hamlin, J. T., Reutter, F. W., and Friend, D. G.: Evaluation of Catechol Amine Levels in Renal Insufficiency, J. Clin. Invest. **37**: 409, 1958.
33. Roth, G. M., and Kvale, W. F.: A Tentative Test for Pheochromocytoma, Am. J. M. Sc. **210**: 653, 1945.
34. Roth, G. M.: Laboratory Diagnosis of Pheochromocytoma, Minnesota Med. **41**: 297, 1958.
35. Gitlow, S. E., Mendlowitz, M., Khassis, S., Cohen, G., and Sha, J.: The Diagnosis of Pheochromocytoma by Determination of Urinary 3-Methoxy,4-hydroxymandelic Acid, J. Clin. Invest. **39**: 221, 1960.
36. Roth, G. M., Flock, E. V., Kvale, W. F., Waugh, J. M., and Ogg, J.: Pharmacologic and Chemical Tests as an Aid in the Diagnosis of Pheochromocytoma, Circulation **21**: 769, 1960.
37. Sjoerdsma, A., Leeper, L. C., Terry, L. L., and Udenfriend, S.: Studies on the Biogenesis and Metabolism of Norepinephrine in Patients With Pheochromocytoma, J. Clin. Invest. **38**: 31, 1959.
38. Goodall, McC., Kirshner, N., and Rosen, L.: Metabolism of Noradrenaline in the Human, J. Clin. Invest. **28**: 707, 1959.
39. Blaschko, H., Hagen, P., and Welch, A. D.: Observation on the Intracellular Granules of the Adrenal Medulla, J. Physiol. **129**: 27, 1955.
40. Falck, B., Hillarp, N.-A., and Högberg, B.: Content and Intracellular Distribution of Adenosine Triphosphate in Cow Adrenal Medulla, Acta physiol. scandinav. **36**: 360, 1956.

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MINERAL COMPOSITION OF HUMAN BONE IN VARIOUS CLINICAL DISORDERS

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Present theoretic concepts of bone hold it to be an active participant in electrolyte and hydrogen-ion homeostasis which engages in ionic exchanges with extracellular fluid. This view derives chiefly from (1) changes in bone sodium, potassium, and carbonate after acute acidosis in animals and (2) the high percentage of exchangeable sodium observed in man and animals with radioisotope dilution studies.

Data on the electrolyte composition of normal bone are scanty. Virtually none are available in human disease states which might be expected to produce changes in the bone "reservoir" of sodium and other electrolytes. In this study cortical bone specimens were obtained from a "control" group of 15 patients who died unexpectedly or were undergoing orthopedic surgical procedures. These were compared with bone from 71 patients who died from a variety of disorders such as infection, cancer, heart disease with and without edema, and renal disease with acidosis.

Average values for sodium, potassium, water, fat, calcium, and phosphorus in human cortical bone were found to be comparable in a wide variety of diseases and largely independent of age, sex, and site of sampling. No correlations existed between the measured bone electrolytes and the serum levels of sodium, chloride, blood urea nitrogen, potassium, and CO_2.

In two patients with uremia and acidosis of long standing, the bone sodium was significantly low. Bone sodium was higher than the control levels in two patients with hypercapnia.

Net changes in bone electrolytes probably occur only with severe and prolonged disturbances of the extracellular fluid components.

THE present study is an attempt to examine some of the current theoretic concepts of the function of human bone as an electrolyte reservoir by the direct examination of its mineral composition in a variety of clinical disorders.

Significant quantitites of sodium, potassium, and other minerals were demonstrated in the skeletal systems of man and other animals by Gabriel in 1894.[1] Since then there have been recurrent suggestions that this relatively large deposit of mineral salts can be readily mobilized in the maintenance of ionic homeostasis. Bone is now considered by many to be a dynamic tissue

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actively participating in normal and abnormal electrolyte metabolism by appropriate ionic exchanges with extracellular fluid.[2-6]

The experimental basis for this "reservoir" concept in man is largely indirect and is derived from three types of evidence: (1) accumulated data on changes in bone sodium, potassium, or carbonate in animals subjected to acid loads, CO_2 inhalation, and electrolyte depletion,[7-14] (2) the demonstration that a significant percentage of the important bone electrolytes are readily "exchangeable" by radioisotope dilution methods,[15-19] and (3) more precise understanding of the ultrastructure and the physicochemical activities of bone.[2, 6, 20-21] Extrapolations from such data have been made to include man and a similar reservoir function has been adduced for this species.[3-5]

Direct information, however, on the mineral constituents of "normal" human bone is scanty.[1, 16, 22-26, 47] There has been no extensive study of the variations in human bone electrolyte content in disease states so that it is not known whether the data on animals are precisely translatable to equivalent states in the human. An attempt is made in this report to study the relation between serum and bone electrolyte concentrations in a number of pathophysiologic states which approximate those produced in experimental animals.

Contrary to some of the indications obtained in experiments on animals, the data to be presented indicate that the individual and average values of the major inorganic constituents of bone are quite constant in a variety of diseases and are generally independent of age, sex, and the serum level of the corresponding electrolyte. Severe chronic metabolic acidosis and hypercapnia from varying causes appear to be correlated with detectable variations in the sodium content of bone. It would seem that present inferences from data on animals regarding the electrolyte reservoir function of human bone may apply in only a limited number of clinical situations.

MATERIAL AND METHODS

A. *Patients.*—Samples of tibia and other bones were examined from a control series of fifteen patients and another group of seventy-one patients who died from a variety of clinical disorders. The control specimens were obtained from patients without significant illness who underwent operation for reduction of fracture or other orthopedic procedure, or from patients who died unexpectedly without antecedent history of illness after trauma or acute myocardial infarction. There were twelve males and three females in this group and their ages ranged from the neonatal period to seventy-four years.

The "abnormal" series consisted of specimens obtained at autopsy from seventy-one patients who died from a variety of clinical disorders. There were forty-nine men and twenty-two women in this group and the age range was from 24 to 91 years, with a mean age of 55 years.

The diagnoses used as the basis for comparison in both groups were determined by gross and histologic postmortem examination. None had histologic evidence of bone disease.

B. *Methods.*—Bone specimens were obtained at operation or at autopsy 2 to 12 hours after death. Most of the samples were removed from the upper one third of the tibia and measured approximately 1½ by 3 cm. A smaller number was obtained from the rib, skull, radius, femur, and phalanges.

The periosteum was carefully stripped away and bone marrow was completely removed. Adherent blood and tissue fluid were removed by blotting, and the specimens were wrapped in

plastic material and stored at 0° C. The cortical portion only of each specimen was reduced to a fine powder with a high speed electric hand drill or, in the case of small specimens, with a dental drill. Care was exercised not to penetrate the inner layers of bone close to the marrow cavity. Bone powder (500 to 1,000 mg.) was obtained, weighed, and then heated to constant weight at 105° C. This was achieved in 48 hours. The dry powder was ashed in a muffle furnace in platinum crucibles at 1,500° F. for 48 hours. The resultant ash was dissolved by adding a small amount (4 to 6 ml.) of 3N hydrochloric acid to the platinum crucible on a hot plate. Solution usually occurred within 15 to 20 minutes, after which the material was filtered through No. 40 filter paper into a 25 ml. volumetric flask. The crucible was washed twice with 3N hydrochloric acid and this was added to the volumetric flask which was made up to volume with distilled water. Ten milliliters of this solution was placed on a Dowex-50 column (50 to 100 mesh) in glass columns (1.3 by 61 cm.) with a sintered glass filter. Fractional elutions were obtained with 0.7N hydrochloric acid by the method of Forbes and D'Ambruso.[27] The eluates were analyzed as follows: the first 100 ml. for phosphorus by the molybdate method,[28] the next 100 to 300 ml. portion for sodium, and the next 300 to 500 ml. portion for potassium. The final elution was made with 5N hydrochloric acid, using a volume of 500 ml., and this was analyzed for calcium by oxalate precipitation and cerate titration.[29] Sodium and potassium were determined by standard methods with the use of an internal standard flame photometer.[30]

Serum electrolyte determinations were obtained at least 24 to 48 hours before death in all patients with significant electrolyte disorders. In other patients with more chronic diseases the determinations were obtained serially at longer intervals before death, which rarely, however, exceeded one week.

Fat was extracted by the method of Folch and collaborators[18] with methanol and chloroform. The determinations were accomplished without fat extraction and are recorded in terms of dry weight, since the fat content was 1.0 per cent or less in 50 specimens—0.703 ± 0.203 per cent.

All determinations were carried out in duplicate. Recorded values are averages. The variation in paired samples did not exceed 2 per cent for sodium, calcium, and phosphorus. Recovery rates of known solutions of sodium, calcium, and phosphorus from the Dowex columns averaged 98 per cent. Recoveries were run before and after each set of determinations. Potassium determinations were more variable and recoveries averaged 95 per cent.

Because of prior observations that a high concentration of calcium, hydrogen ion, or phosphorus would interfere with the determination of sodium in the flame photometer,[24, 30-31] fifty samples were examined before and after passage through the Dowex column.

RESULTS

1. *Water Content.*—The water content of the cortical bone specimens ranged from 5.2 to 11.7 per cent, with an average of 8.7 ± 0.20 per cent. There was no appreciable variation either with the age of the patient or with the presence or absence of disease.

2. *Potassium.*—The range of concentration for potassium was between 5 and 20 μEq per gram of dry bone with an average value of 15 μEq. The levels of bone potassium were considered too low and the recoveries too variable to permit reliable comparison between the control determinations and those obtained in disease states.

3. *Comparison of Data Before and After Dowex-50 Column.*—Table I gives the comparison of values obtained for sodium before and after passage of the ashed sample dissolved in hydrochloric acid through the Dowex-50 column. The mean of paired direct determinations of sodium on the dissolved ash in forty-four patients was 273 μEq per gram of dried bone. The mean of paired

TABLE I. COMPARISON OF BONE SODIUM DETERMINATIONS IN 50 PATIENTS BEFORE AND AFTER PASSAGE THROUGH DOWEX-50 COLUMN

	BEFORE PASSAGE THROUGH COLUMN	AFTER PASSAGE THROUGH COLUMN
Mean	272 μEq/Gm.	261 μEq/Gm.
S.E.	±2.2	±2.2

determinations of sodium on the eluate after passage through the Dowex-50 column was 261 μEq per gram. Values obtained by the direct determination were on the average 11 mg. higher than those determined after passage through the ion exchange column.

4. *Control Series.*—Table II shows the results obtained in the specimens from the control series of fifteen patients. Six patients died unexpectedly as a result of myocardial infarction. Four of these patients were dead on arrival at the hospital. Two patients died as the result of trauma. The tibias of one premature infant and one stillborn infant were examined.

TABLE II. INDIVIDUAL VALUES FOR NA, CA, AND P IN 15 CONTROL SPECIMENS OF HUMAN BONE OBTAINED AT OPERATION OR AUTOPSY

DIAGNOSIS	AGE (YR.)	SEX	SITE	NA (μEQ/GM.)	CA (MEQ./GM.)	P (MG./GM.)
Autopsy.—						
Myocardial infarction; dead on arrival	74	M	Tibia	273	10.8	111
Myocardial infarction; dead on arrival	37	F	Tibia	242	11.3	108
Myocardial infarction; dead on arrival	72	M	Tibia	271	11.7	118
Myocardial infarction; dead on arrival	39	M	Tibia	273	11.1	109
Myocardial infarction	55	M	Tibia	266	10.9	112
Myocardial infarction	66	M	Tibia	272	10.3	108
Skull fracture	60	M	Tibia	274	10.2	108
Trauma	63	F	Skull	248	10.2	90
Prematurity	1 day	M	Tibia	258	7.4	102
Stillborn	—	M	Tibia	258	9.1	113
Removed at Operation.—						
As gangrene	53	M	Tibia	246	9.4	102
Compound fracture	29	M	Tibia	250	10.0	106
Compound fracture	30	F	Radius	270	—	—
Bunion	39	M	Metatarsal	270	9.9	106
Traumatic amputation	63	M	Radius	260	10.7	110
Mean determination of control ± S.E.				262 ± 2.8	10.2 ± 0.30	107 ± 1.7

Five specimens were removed at operation. The specimen obtained most frequently was from the tibia, but samples of skull, radius, and metatarsal were also examined.

The range of bone sodium in this group was from 242 to 273 μEq per gram of dry bone and the mean value was 262 μEq per gram. The range of phosphorus was 90 to 118, with a mean of 107 mg. per gram of dry bone. The range of calcium was 7.4 to 11.7, with a mean of 10.2 mEq. per gram of dry bone. The bone calcium was significantly low in the premature infant, though it was close to normal in the stillborn infant. The bones of the premature infant and the stillborn infant showed normal values for sodium and phosphorus. Though

most specimens were from the tibia, the values for sodium, calcium, and phosphorus and also potassium and water were not different in the radius, metatarsal, or skull in the control group.

Table III shows the mean of the determinations for sodium, calcium, and phosphorus by age for the control series. The number of observations is small, but there appears to be a slight increase of bone sodium with age. No detectable variation can be made out in the calcium and phosphorus values, except as already noted in the premature infant.

TABLE III. MEAN DETERMINATION OF NA, CA, AND P BY AGE FOR CONTROL SERIES

AGE	NO.	NA (μEQ/GM.)	CA (MEQ./GM.)	P (MG./GM.)
Newborn	1	258	9.1	113
Premature	1	258	7.4	102
20-29	1	250	10.0	106
30-39	4	263	10.1	107
40-49	0	—	—	—
50-59	2	256	10.1	107
60-69	4	263	10.3	104
70-79	2	272	11.2	115

Heart Disease.—Table IV shows the average values obtained in patients who died from heart disease of different etiologies. There were seventeen patients with arteriosclerotic, hypertensive, or rheumatic heart disease. Three of these patients were edematous at the time of death and fourteen were free of edema. Of the latter fourteen, ten had been edematous, were treated, and were edema-free at the time of death. Two of these patients had serum sodium values below 130 mEq. per liter and chloride values below 90 mEq. per liter. The others had normal serum electrolytes. The average bone sodium for all fourteen patients in this category was 259 μEq per gram of dry bone with no significant differences between the edematous and the nonedematous patients.

TABLE IV. MEAN VALUES OF BONE NA, CA, AND P IN PATIENTS WITH HEART DISEASE OF VARIOUS TYPES

HEART DISEASE	NO. OF CASES	NA (μEQ/GM.)	CA (MEQ./GM.)	P (MG./GM.)
Arteriosclerotic, hypertensive, rheumatic	17	259	10.0	107
Edema at death	3	254	10.6	109
No edema at death	14	260	10.1	106
Cor pulmonale	4	256	9.7	110
Amyloid edema	1	258	9.6	110

There were four patients with cor pulmonale who had extensive edema at the time of death. Two of these had low serum sodium and chloride values. The bone sodium, calcium, and phosphorus levels were not appreciably different from the controls.

One of the patients studied had widespread myocardial amyloidosis with anasarca. His bone sodium was also within the normal range.

Thus, the comparison of eight patients with heart disease, edematous at death, with seventeen nonedematous patients with heart disease revealed no

significant differences in the average levels of bone sodium, calcium, or phosphorus.

Miscellaneous Diseases.—Table V shows the data on specimens obtained from patients who died from a variety of disorders. There were fourteen patients in this group with malignant neoplasms. Most of these patients were cachectic and had been ill for long periods. Bone sodium, calcium, and phosphorus determinations were not significantly altered from the control data. Five had clinical edema at the time of death. All of these patients had normal serum electrolytes except for two patients with low serum sodium and chloride values.

TABLE V. MEAN VALUES FOR Na, Ca, AND P IN PATIENTS WITH MISCELLANEOUS DISEASES

DISEASE	NO. OF CASES	Na (μEq/Gm.)	Ca (mEq./Gm.)	P (mg./Gm.)
Malignant disorders	14	259	10.5	115
Infectious diseases	6	259	10.5	109
Other medical	5	265	10.1	112
Perforated viscus	7	263	11.0	111
Control		261	10.4	107

Six patients were studied who died from infectious diseases. Four of these were elderly persons with staphylococcal pneumonia. Two of the latter had low serum chloride and sodium values.

Five patients with varying diseases, one of whom had low serum sodium and chloride levels had no significant changes in bone electrolytes. In this group there were five patients with various diseases: subarachnoid hemorrhage, muscular dystrophy, third degree burns, cerebral vascular accident, and pulmonary fat embolization following hip fracture.

TABLE VI. INDIVIDUAL VALUES FOR SERUM AND BONE ELECTROLYTES IN PATIENTS WITH HYPERCAPNIA AND VARIOUS CLINICAL DISORDERS

	SERUM			BONE		
DIAGNOSIS	CO_2	Cl	Na	Na (μEq/Gm.)	Ca (mEq./Gm.)	P (mg./Gm.)
Hypertension and arteriosclerotic heart disease	49	105	151	300	10.9	—
Cancer of ovary	40	93	134	243	11.1	—
Cancer of bile ducts	41	102	135	300	11.0	148
Cor pulmonale	41	92	158	253	9.6	103
Hypertension and arteriosclerotic heart disease	35	94	—	242	10.0	111
Cancer of lung	37	75	—	256	10.3	103
Cor pulmonale	34	83	—	252	10.7	111
Average				264	10.5	115
Control				261	10.4	107

Seven of the specimens from this miscellaneous group were obtained from patients who died before or shortly after surgical exploration as a result of perforation of abdominal viscus with peritonitis. All of these patients were elderly people admitted late in the course of their illness. All had normal serum electrolytes. Blood urea nitrogen values were moderately elevated in all. In two patients, carbon dioxide combining power was slightly depressed.

TABLE VII. INDIVIDUAL SERUM AND BONE ELECTROLYTES IN PATIENTS WITH ACIDOSIS DUE TO RENAL DISEASE*

PATIENT	DIAGNOSIS	SERUM			BONE		
		BUN	Na	CO_2	Na (μEq/Gm.)	Ca (mEq./Gm.)	P (mg./Gm.)
H. J.	Glomerular nephritis	173	130	4.8	219	--	--
W. L.	Malignant nephrosclerosis	160	131	18.0	200	11.9	153
G. J.	Chronic pyelonephritis	57	144	14.0	264	10.8	108
P. S.	Chronic pyelonephritis	73	138	15.0	252	10.7	108
M. P.	Acute tubular necrosis	266	158	13.0	250	10.1	114
L. T.	Chronic pyelonephritis	115	142	14.0	270	11.0	110
R. R.	Massive infarction of kidney	98	142	14.0	253	10.8	--
P. G.	Hepatic coma; metabolic alkalosis	114	136	8.4	277	11.6	111
M. T.	Duodenal fistula	116	150	48.0	275	10.1	99

*One patient with metabolic alkalosis is added for comparison.

The average values for bone sodium, calcium, and phosphorus were similar to those in the control series for all patients in this miscellaneous group.

Hypercapnia.—Table VI shows the data on a group of patients who had a significant degree of hypercapnia for at least one week prior to death. All these patients exhibited evidence of diminished pulmonary ventilation. The primary diagnoses varied. Three patients died from carcinomas, one of the ovary, one of the bile duct, and one of the lung. Two patients had cor pulmonale and two had hypertensive and arteriosclerotic heart disease. These five patients were not included in the group already presented in Tables III and IV. In all cases the serum carbon dioxide level was elevated above 34 mEq. per liter. Serum chloride level was below 90 mEq. per liter in all except two. Serum sodium was normal in two and greater than 145 mEq. in two. The bone sodium was well within the normal range in five and definitely elevated in two. Bone calcium and phosphorus were also within the normal range except for a high phosphorus in one patient with carcinoma of the bile duct.

Renal Disease With Acidosis.—Table VII presents the individual data for eight patients who died as a result of acidosis due to renal insufficiency. Blood urea nitrogen, serum sodium, and carbon dioxide combining power levels were those obtained on the day of death. In all instances, they represent values obtained after therapy directed at the alleviation of acidosis and electrolyte disturbances. The first two patients listed (H. J. and W. L.), were middle-aged men who died from chronic glomerulonephritis and malignant nephrosclerosis, respectively. They were known to be acidotic for at least 3 months before death and both had relatively low serum sodium and chloride values. The carbon dioxide combining power was reduced in both and the whole blood pH was 7.0 or below at death. Bone sodium levels were significantly depressed below the mean control values for both patients. Bone calcium was determined in only one of these two patients and was found to be normal. The bone phosphorus was elevated in W. L.

Five other patients demonstrated acidosis due to renal disease of relatively shorter durations. Patients G. J., P. S., and L. T. had bilateral severe pyelonephritis. Patient M. P. had acute tubular necrosis, following extensive third degree burns, and patient R. R. was found to have massive infarction of one kidney, after spontaneous thrombosis of the renal artery. Another patient, P. G., was in hepatic coma and severe acidosis at the time of death. The histologic lesion in his kidney was that of benign nephrosclerosis and chronic pyelonephritis. All of these patients exhibited azotemia and depression of the carbon dioxide combining power, with normal serum sodium values.

Bone sodium, calcium, and phosphorus were not significantly altered from the mean value for the controls. The mean bone sodium for all patients with metabolic acidosis was 244 μEq per gram of dry bone. The low mean value is due to the inclusion of H. J. and W. L.—two patients with acidosis of long standing.

For purposes of comparison there are included the data on one patient (M. T.) with severe metabolic alkalosis due to an external duodenal fistula with gastroduodenal continuity. This patient died with azotemia, hypochloremia, kypokalemia, and elevated blood pH. His bone sodium was within the normal range, calcium was normal, and phosphorus was at the lower limits of normal. This patient had no evidences of renal disease at autopsy.

Correlation of Serum and Bone Sodium.—Fig. 1 is a plot of the serum sodium values in the "abnormal" series on the ordinate against the bone sodium in microequivalents per gram of dry bone on the abscissa. The broken lines on the x axis represent the range of two standard deviations of the mean for bone sodium and, on the y axis, the normal range of serum sodium values in this laboratory. There is no apparent linear correlation of bone sodium with the corresponding serum sodium values. Nine patients with serum sodium values below 135 mEq. per liter can be seen to fall within the normal range for bone sodium and, conversely, six patients with serum sodium values above 150 mEq. per liter are seen to fall also within the normal range. The two very low bone sodium values are those in the two patients (W. J. and H. L.) with chronic acidosis due to renal disease. Two patients with bone sodium values of 158 mEq. per liter were in the group with hypercapnia.

TABLE VIII. MEAN DETERMINATIONS WITH STANDARD ERRORS

DIAGNOSIS	Na (μEq/Gm.) NO. OBSERVATIONS	Na (μEq/Gm.) MEAN ± S.E.	Ca (mEq./Gm.) NO. OBSERVATIONS	Ca (mEq./Gm.) MEAN ± S.E.	P (mg./Gm.) NO. OBSERVATIONS	P (mg./Gm.) MEAN ± S.E.
Control	15	262 ± 2.8	14	10.2 ± 0.30	14	107 ± 1.7
Renal disease						
Acidosis	7	244 ± 9.5	6	10.9 ± 0.22	5	119 ± 8.7
Metabolic alkalosis	1	275	1	10.1	1	99
Hypercapnia	7	264 ± 9.6	7	10.5 ± 0.20	5	115 ± 9.1
Heart disease	22	259 ± 4.9	20	10.0 ± 0.17	18	108 ± 1.8
Malignant disorders	14	260 ± 6.1	13	10.5 ± 0.32	11	115 ± 5.8
Infectious diseases	6	259 ± 4.4	5	10.5 ± 0.20	5	109 ± 1.7
Other medical	5	266 ± 2.4	4	6.7 ± 0.66	4	113 ± 2.4
Perforated viscus	7	262 ± 4.0	6	11.0 ± 0.17	5	111 ± 0.6

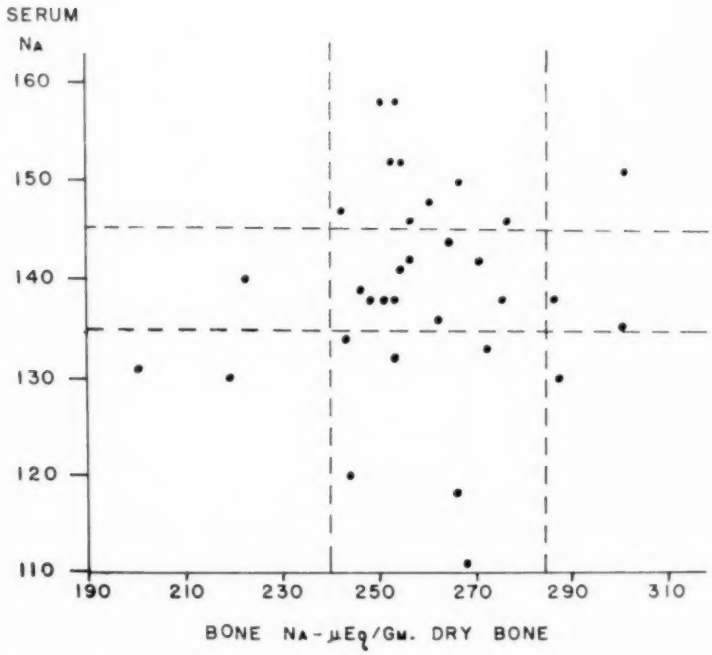

Fig. 1.—Serum sodium plotted against corresponding bone sodium value in thirty-two patients.

Correlation of Serum CO_2 Combining Power and Bone Sodium.—Fig. 2 shows a linear plot of the bone sodium content in microequivalents per gram of dry bone against the corresponding serum carbon dioxide combining power in milliequivalents per liter. There is no evidence of a linear trend y on x from these data. The reduction in variance due to regression of y on x is virtually nil.

Summary of Data.—Table VIII is the summary of the mean determinations with standard errors for sodium, calcium and phosphorus in bone specimens from the control series compared with the abnormal series. There is very little difference between the mean values for sodium in the control subjects and in the patients in various disease categories. The low mean recorded for the patients with renal disease and acidosis is explained entirely on the basis of the two low bone sodium values which were detected in patients with long standing acidosis. There are no significant variations in calcium and phosphorus between the control series and the patients in various disease categories. A simple "between-within" analysis of variation indicates that most of the variability in data is due to individual fluctuations rather than to differences among groups.

Comment on Methods.—A number of investigators have commented upon the problems encountered in the flame photometric determination of sodium in the presence of calcium in solutions of ashed bone.[27, 30, 32] The difficulties have been attributed to the closeness of the absorption spectra of calcium and

sodium,[30-31] to the high acid concentration of bone ash solutions, to the presence of contaminating substances such as ammonia, phosphate, iron, and oxalate, and finally to coprecipitation of sodium with calcium if the sodium determination is done after the calcium determination.[24] Others[12, 33] have not encountered these difficulties or have been able to obtain satisfactory analyses of sodium in bone ash by variations in the technique of calcium precipitation[11] or by suitable corrections.[16]

Our data comparing fifty analyses performed on the bone ash solution before and after passage through the ion exchange column reveal a mean difference of 11 mEq. of sodium per kilogram of dry bone. Direct determination resulted in uniformly higher values. Calcium and phosphorus on the other hand were determined before and after passage through the column and no significant differences in the two methods were observed. Insufficient data were obtained on a comparison of potassium by the two methods. It is our impression that sodium and potassium in acid–bone ash solution should be determined by passage through ion exchange columns as already suggested.[27, 32] However, calcium and phosphorus determinations can be made directly with no significant differences in results.

The tibia was chosen in the present study because it presented a compact bone which is easily removed at surgical operation. Specimens of adequate size could be obtained from approximately the same site in different patients. Furthermore, marrow could be easily removed. Attempts to obtain suitable specimens of cortex from ilium, rib, or vertebra were unsuccessful since there was almost inevitable contamination with bone from the marrow cavity. Levels

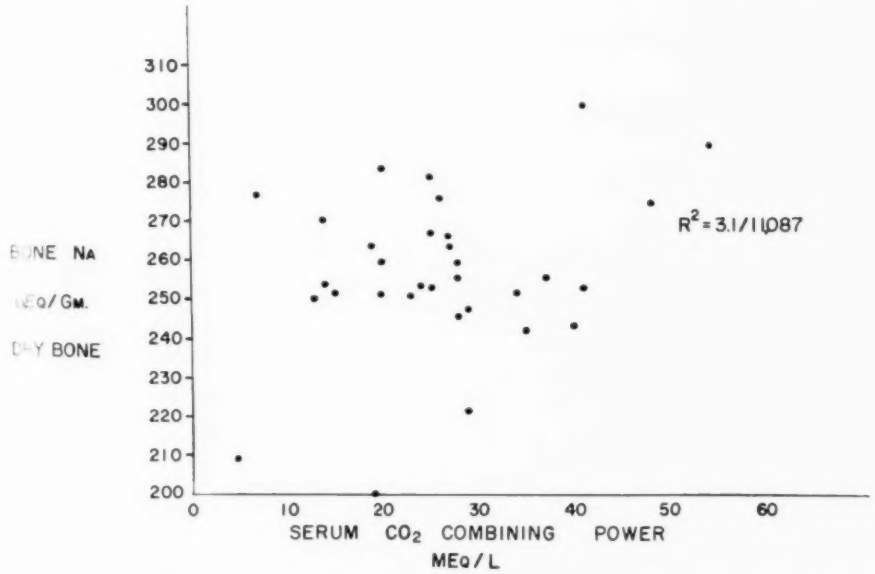

Fig. 2.—Serum CO_2 combining power plotted against corresponding bone sodium in thirty-two patients.

of sodium for bones from these latter sites were considerably lower than those obtained from the tibia. The importance of completely removing marrow from the bone specimens has been pointed out by Follis[22] who showed consistently higher levels of calcium, phosphorus, and carbonate in marrow-free preparations of human rib when compared with specimens which contained marrow. Our data would indicate that the tibia is a technically satisfactory site for sampling and that it gives reproducible values without the problem of contamination from marrow elements.

The data for the mineral composition of several human bones determined by different investigators has been summarized recently.[26]

Our values for calcium and phosphorus are in close agreement with those of other investigators.[16, 22, 25-26, 34]

The range of potassium values obtained in this study is from 10 to 20 μEq per gram of dry bone. This agreed with the range of values reported for skull, but was lower than the values reported for rib and ilium.[26]

Our data on the sodium content of human bone also agree quite closely with other results obtained recently. Thus, Agna and his colleagues[26] found 291 mEq. of sodium per kilogram of dry bone in skull, 268 in rib, and 269 in ilium. Edelman and co-workers[16] reported values of 273 mEq. in skull, 215 in rib, and 222 in ilium. These figures compare well with our normal control value of 262 mEq. per kilogram for tibia. Both of these studies indicated a higher level of bone sodium in skull than in the rib and ilium. Values as low as 222 mEq. per kilogram were reported for ilium by Edelman and associates.[16] There was closer agreement between the three bones in the study of Agna and colleagues.[26] The earliest study by Gabriel[1] revealed a level of 251 mEq. per kilogram of dry bone in two humeri and this accords very well with the recently obtained data. Somewhat lower levels for rib were reported by Davies and collaborators.[24] They found a rather high level for femur (300 mEq. per kilogram of dry bone).

Generally speaking, the lower levels reported are for cancellous bones like rib and ilium.[26] No data on human tibiae were available for comparison.

DISCUSSION

If we accept the mean values of this and other studies for the sodium content of human bone, the estimated total skeletal content of this cation would be approximately 1,500 to 3,000 mEq. Were this available for exchange with extracellular fluids, bone would constitute an important line of defense in disturbances of hydrogen ion balance and in systemic hyponatremia and hypernatremia.

Over the past 60 years animal studies have indicated varying degrees of participation of the mineral constituents of bone in homeostasis under different pathophysiologic stresses. As early as 1893, Gusmitta[7] showed a decrease in bone carbonate in rats with inanition acidosis. Goto,[8] in 1915, fed relatively large loads of hydrochloric acid to rabbits and reported a significant decrease in bone potassium and carbonate. In 1932, Irving and Chute[9] demonstrated

a 12 per cent decrease in bone carbonate in rats and guinea pigs after ingestion of ammonium chloride. Their results were not conclusive so far as phosphorus and calcium were concerned, but they suggested that bone could function as a buffer reserve in the neutralization of ingested acid. Irving and co-workers,[35] in 1934, obtained suggestive evidence that bone could act as a site of carbon dioxide retention in animals subjected to CO_2 inhalation. In 1936, Harrison and associates[36] introduced the notion of "extra" sodium in bone and cartilage in excess of what would be expected on the basis of its extracellular fluid content. They felt that this sodium was either insoluble or undissociated, but that it might be available under certain circumstances.

Experimental data have been obtained more recently relating specifically to the participation of bone sodium and potassium in electrolyte shifts. Juvenile rats responded to the administration of an acid load with equimolar losses of sodium, potassium, and carbonate.[10] Other studies subjecting rats to acid loads in association with peritoneal dialysis resulted in average loss of bone sodium of 42 mEq. per kilogram and of bone potassium of 11 mEq. per kilogram.[11] Levitt[12] administered large loads of ammonium chloride and hydrochloric acid to rats and showed losses of bone sodium of the same order of magnitude.

In a recent review, Nichols and Nichols[3] refer to data of their own indicating significant losses of bone sodium in adult dogs undergoing sodium depletion and an increase in bone sodium in adult rats with chronic sodium loading. Likewise, hyperchloremic acidosis in adult rats was shown to produce a loss of bone sodium. In rats on a low sodium diet there was a correlation of bone sodium with urinary sodium losses after administration of Mercuhydrin.[14] Two studies on bone sodium in adrenalectomized rats show a slight decrease.[33, 37]

Two of the most recent animal studies, however, have failed to confirm some of these changes in bone electrolyte composition. Winters and collaborators[38] produced severe hyponatremia in adult male rats with marked depression of serum and sodium values, but were not able to demonstrate any significant transfer of sodium from bone under these circumstances. Levitin and associates[39] investigated the effect of respiratory acidosis on electrolytes of liver, muscle, and bone. They were able to demonstrate some participation of potassium proteinate of muscle in the buffering of carbonic acid when the animals inhaled 8 per cent carbon dioxide, but observed no changes in bone sodium. Forbes and co-workers[40] were recently able to show only small to moderate changes in bone sodium in adult cats with acidosis. The magnitude of these changes did not correlate with degree of acidosis, extracellular fluid sodium, or amount of acid infused.

In marked contrast with animal data of the type summarized above, there is virtually no direct information on the changes in human bone electrolyte composition in clinical states which might parallel the animal experiments. Bergstrom[5] alludes to bone analyses in human acidosis but does not give data or references.

Certainly, the present views of the microstructure of bone crystals provide a strong theoretic foundation for the expectation that adsorption, substitution, and interchanges of important ions occur at the crystal surface.[2, 6, 20-21] Such

surface phenomena would require no distortion of internal crystal lattice structure and would provide an excellent setting for exchange of important ions in response to changes in the extracellular fluid concentration.

Moreover, bone sodium and potassium are promptly and significantly exchangeable with their respective radioisotopes. Thus, 35 to 45 per cent of the total skeletal sodium has been found to be exchangeable in man[15, 16] and dog[16] and as high as 69 per cent in the rabbit.[23] These figures would fit well with the earlier estimates of Harrison and co-workers[36] that one third of total body sodium was associated with bone salts. However, except for the studies of Bergstrom[10-11] and Levitt,[12] the magnitude of the changes observed even in the animal experiments is well below what might be predicted from the figures on exchangeable sodium.

The present study indicates that exchangeable sodium of bone is in reality not readily available in the majority of patients with a variety of disorders which theoretically would be expected to call upon this reserve. Even in the two patients with chronic severe acidosis (W. L. and H. J.) the bone sodium was reduced only 12.5 and 23.7 per cent respectively—well below the 45 per cent total exchangeable sodium. In these two cases, the conclusion appears reasonable that bone sodium ion did partake in exchange with hydrogen ion as a response to metabolic acidosis. Thus, some direct evidence is provided that bone may partake with other tissues in the buffering of hydrogen ion as suggested in nephrectomized dogs and in the normal human with ammonium chloride acidosis.[45-46] Bone calcium in all cases of acidosis studied was normal, though it is well known that in prolonged acidosis this mineral may be severely depleted in bone. It may be that other factors, i.e., parathyroid overactivity, longer duration of acidosis, etc., are necessary to effect changes in bone calcium in acidosis. Thus, it appears, as has been emphasized already,[3] that "exchangeable" and "available" sodium may not be physiologically identical entities. The equilibrium demonstrated by radioisotope dilution between extracellular fluid and bone electrolytes may be difficult to displace in either direction except under very stressful circumstances.

There is not sufficient evidence in this study to permit conclusions with regard to the effect of metabolic alkalosis on the ionic constituents of bone. Only one patient in this category was studied and here the bone sodium, calcium, and phosphorus were not significantly affected.

Some of the disparity between the results in most of the patients with acidosis studied here and the data in rats may be attributable to the facts that the magnitude of the acid load and the acuteness of the experiments were not precisely paralleled in any of the clinical material. Species differences and the relatively young age of the rats may also be important, since the amount of exchangeable sodium varies in different animals[23-24] and the activity of the osteones declines with age.[4, 21] Further, in the animal data, hyponatremia and acidosis were common denominators, whereas this was so only in our two patients who did satisfy theoretic expectations.

A recent study has demonstrated an increase in total body sodium and a linear relation between serum CO_2 and exchangeable sodium in patients with

respiratory acidosis.[40] Two of the patients presented here who had hypercapnia and high serum CO_2 combining power showed rather high bone sodium values. It is conceivable that under these conditions sodium may enter bone. There was, however, no linear correlation between serum carbon dioxide and bone sodium. Our data would indicate that in chronic pulmonary insufficiency some portion of this total exchangeable sodium may be present in bone, but that this is not invariably so.

It has long been recognized that there is diminished excretion of sodium in congestive heart failure. An increase in total body sodium in patients with congestive heart failure with edema has been demonstrated[41] and in cardiac patients without edema[42] as well. In the presence of edema, the retention of total body sodium is out of proportion to the retention of water. Analysis of the sodium content of a number of organs in patients who died from congestive heart failure shows a particularly marked increase in the renal cortex.[43] Theoretically it might be expected that some of this retained sodium would be found in bone. However, the present study failed to demonstrate any significant increase in sodium in the bones of patients with heart disease whether or not they had edema.

The present study does not provide any additional information in the understanding of the altered physiology in the hyponatremia which occurs in congestive heart failure and in other chronic illnesses. As is well known, hyponatremia may be associated with dilution of body fluids and retention of sodium or it may be due to excess loss of sodium. Balance studies during the development of hyponatremia in patients with cardiac disease have re-emphasized the complexity of internal redistribution of cations in this disease.[44] Patients in this series who had serum hyponatremia together with edema and would, therefore, be expected to have high total body sodium did not reveal any changes in the bone sodium. This study would indicate that tissues other than bone are the sites of sodium retention.

On the basis of present theoretic concepts about the function of bone, it is suspected that in certain other diseases bone sodium content might be affected. Thus, in long-standing chronic illnesses, like malignancy with malnutrition and decreased intake of food and electrolytes, serum hyponatremia often occurs. Depletion of bone sodium was not observed under these circumstances. Likewise, electrolyte disturbances, particularly hypochloremia and hyponatremia are known to occur in both acute and chronic infections. In this study there were no associated changes found in bone sodium.

There were a number of patients with hypernatremia in this series. Contrary to what would be expected from sodium loading experiments in animals, there was no demonstrable increase in bone sodium under these circumstances. The site of retention of excess sodium probably occurred in tissues other than bone.

The general failure of the data in this study to show a significant correlation between bone and serum sodium in a variety of patients with extracellular fluid and electrolyte disturbances appears to challenge some of our present

concepts of a free ionic interchange between these phases in the human. Available sodium and exchangeable sodium are probably not identical and the bone sodium may be closely bound to the crystalline phase and relatively unavailable as predicted some years ago.[36] Further, there is evidence that while the uptake of radiosodium by bone is rapid, its mobilization may be very slow.[21] Radioisotope activity is known to vary from bone to bone and from site to site in the same bone.[21]

Likewise there is a loss of crystal surface and osteone activity with increasing age[4, 21] and it may be that bone electrolyte shifts will be more important in infants and children than they appear to be in adults.

The tibial cortex may be less active than that of other bones, although there was no real difference in bone sodium in other sites. Further, the bone sodium, calcium, phosphorus, and potassium content was found to be identical at different levels in the same specimen so that site variations within the same bone do not appear likely.

Further data are undoubtedly needed, particularly in children, in patients with alkalosis, more prolonged acidosis and endocrine disturbances, etc. in order to examine further the dynamic functions of bone. Serial studies employing biopsy techniques[29] in patients with fluid, electrolyte, and acid-base disturbances may prove to be helpful in improving our understanding of the degree of bone participation in electrolyte metabolism.

Further data in our laboratory on the magnesium, citrate, carbonate, and chloride content of human bone likewise show no significant variations in a wide variety of pathophysiologic states.[50]

SUMMARY

Direct determination of the mineral content of tibial cortex was carried out on specimens from a group of fifteen patients who died unexpectedly or who underwent orthopedic procedures. The mean values per gram of dry bone were: sodium, 262 μEq, calcium, 10.2 mEq., phosphorus, 107 mg., and potassium, 15 μEq. These levels were in close agreement with the results obtained in other recent investigations.

Sixty-nine specimens were similarly examined from patients who died from a variety of clinical disorders—heart disease, renal disease with acidosis, hypercapnia, malignant disorders, infections, acute abdominal emergencies, and miscellaneous diseases. In most of the diseases studied, there were no marked or consistent variations from the control data. In the case of severe chronic acidosis and in hypercapnia, there may be significant transfers of sodium between the extracellular fluid and bone marrow.

There was no evident correlation between the level of bone sodium and the corresponding level for serum sodium or serum carbon dioxide combining power, except in two instances where hyponatremia and acidosis were combined.

Levels of bone calcium, phosphorus, and water were not altered appreciably in any of the patients studied, except for high phosphorus determination in one patient with chronic acidosis.

The implication of these data for the present theoretic formulations of the mineral reservoir function of bone are examined. Evidence is adduced that somewhat modifies the existing notion that bone is an easily available source of electrolytes in normal mineral metabolism and in clinical states characterized by electrolyte depletion and disturbances of acid-base balance.

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REFERENCES

1. Gabriel, S.: Chemische Untersuchungen über die Mineralstoffe der Knochen und Zahne, Ztschr. f. physiol. Chem., Strassb. **18**: 257, 1894.
2. Neuman, W. F., and Neuman, M. W.: The Chemical Dynamics of Bone Mineral, Chicago, 1958, University of Chicago Press.
3. Nichols, G., Jr., and Nichols, N.: The Role of Bone in Sodium Metabolism, Metabolism **5**: 438, 1956.
4. Neuman, W. F., and Neuman, M. W.: Emerging Concepts of the Structure and Metabolic Functions of Bone, Am. J. Med. **22**: 123, 1957.
5. Bergstrom, W. H.: The Skeleton as an Electrolyte Reservoir, Metabolism **5**: 433, 1956.
6. McLean, F. C.: The Ultrastructure and Function of Bone, Science **127**: 451, 1958.
7. Gusmitta, M.: Sur les alterations des os produites par l'inanition, Arch. ital. de biol. **19**: 220, 1893.
8. Goto, K.: Mineral Metabolism in Experimental Acidosis, J. Biol. Chem. **36**: 355, 1918.
9. Irving, L., and Chute, A. L.: Participation of Carbonates of Bone in Neutralization of Ingested Acid, J. Cell. & Comp. Physiol. **2**: 157, 1932.
10. Bergstrom, W. H.: The Relationship of Sodium and Potassium to Carbonate in Bone, J. Biol. Chem. **206**: 711, 1954.
11. Bergstrom, W. H., and Wallace, W. M.: Bone as a Sodium and Potassium Reservoir, J. Clin. Invest. **33**: 867, 1954.
12. Levitt, M. F., Turner, L. B., Sweet, A. Y., and Pandiri, D.: The Response of Bone, Connective Tissue and Muscle to Acute Acidosis, J. Clin. Invest. **35**: 98, 1956.
13. Bergstrom, W. H.: The Participation of Bone in Total Body Sodium Metabolism in the Rat, J. Clin. Invest. **34**: 997, 1955.
14. Nichols, N., and Nichols, G.: The Relation of Bone Sodium to Sodium Excretion in Mercurial Diuresis, Clin. Res. Proc. **3**: 44, 1955.
15. Kaltreider, N. L., Meneely, G. R., Allen, J. R., and Bale, W. F.: Determination of Volume of Extracellular Fluid of Body With Radioactive Sodium, J. Exper. Med. **74**: 569, 1941.
16. Edelman, I. S., James, A. H., Baden, H., and Moore, F. D. Electrolyte Composition of Bone and the Penetration of Radiosodium and Deuterium Oxide Into Dog and Human Bone, J. Clin. Invest. **33**: 122, 1954.
17. Manery, J. F., and Bale, W. F.: Penetration of Radioactive Sodium and Phosphorus Into Extra- and Intra-cellular Phases of Tissues, Am. J. Physiol. **132**: 215, 1941.
18. Forbes, G. S., and Perley, A.: Estimation of Total Body Sodium by Isotopic Dilution. I. Studies on Young Adults, J. Clin. Invest. **30**: 558, 1951.
19. Corsa, L., Jr., Olney, J. R., Jr., Steenburg, R. W., Ball, M. R., and Moore, F. D.: Measurement of Exchangeable Potassium in Man by Isotope Dilution, J. Clin. Invest. **29**: 1280, 1950.
20. Robinson, R. A.: Electron Micrography of Bone *in* Reifenstein, E. C., and Brownfield, C., editors: Metabolic Interrelations: Transactions of the Third Conference, New York, 1951, Josiah Macy, Jr., Foundation, p. 271.
21. Neuman, W. F., and Neuman, M. W.: The Nature of the Mineral Phase of Bone, Chem. Rev. **53**: 1, 1953.
22. Follis, R. H., Jr.: The Inorganic Composition of the Human Rib With and Without Marrow Elements, J. Biol. Chem. **194**: 223, 1952.
23. Davies, R. E., Kornberg, H. L., and Wilson, G. M.: Relation Between Total and Exchangeable Sodium in the Body, Nature, London **170**: 979, 1952.
24. Davies, R. E., Kornberg, H. L., and Wilson, G. M.: The Determination of Sodium in Bone, Biochem. J. **52**: xv, 1952.

25. Forbes, R. M., Cooper, A. R., and Mitchell, H. H.: The Composition of the Adult Human Body as Determined by Chemical Analysis, J. Biol. Chem. **203:** 359, 1953.
26. Agna, J. W., Knowles, H. C., and Alverson, G.: Mineral Content of Normal Human Bone, J. Clin. Invest. **37:** 1357, 1958.
27. Forbes, G. B., and D'Ambruso, M.: Determination of Sodium in Bone With the Aid of Cation Exchange Chromatography, J. Biol. Chem. **212:** 655, 1955.
28. Fiske, C. H., and SubbaRow, Y.: The Colorimetric Determination of Phosphorus, J. Biol. Chem. **66:** 375, 1925.
29. Vogt, J. H.: Ash Content of the Spongy Substance of the Iliac Crest, Acta med. scandinav. **135:** 221, 1949.
30. Wallace, W. M., Holliday, M., Cushman, M., and Elkinton, J. R.: The Application of the Internal Standard Flame Photometer to the Analysis of Biologic Material, J. LAB. & CLIN. MED. **37:** 621, 1951.
31. Parks, T. D., Johnson, H. O., and Lykken, L.: Errors in the Use of the Model 18 Perkin-Elmer Flame Photometer for the Determination of Alkali Metals, Anal. Chem. **20:** 822, 1948.
32. Norman, N., Beck, J. C., and Browne, J. S. L.: Separation of Bone Sodium, Potassium, and Calcium by the Use of Cation Exchange Resin, J. LAB. & CLIN. MED. **50:** 308, 1957.
33. Stern, T. N., Cole, V. V., Bass, A. C., and Overman, R. R.: Dynamic Aspects of Sodium Metabolism in Experimental Adrenal Insufficiency Using Radioactive Sodium, Am. J. Physiol. **164:** 437, 1951.
34. Baker, S. L., Butterworth, E. C., and Langley, F. A.: Calcium and Nitrogen Content of Human Bone Tissue Cleaned by Micro-dissection, Biochem. J. **40:** 391, 1946.
35. Irving, L., Ferguson, J. K. W., and Plewes, F. B.: Source of Carbon Dioxide Expired and the Site of Its Retention, J. Physiol. **69:** 113, 1930.
36. Harrison, H. E., Darrow, D. C., and Yannet, H.: Total Electrolyte Content of Animals and Its Probable Relation to Distribution of Body Water, J. Biol. Chem. **113:** 515, 1936.
37. White, H. L., and Rolf, D.: Tissue Electrolytes in Normal and Adrenalectomized Rats, Fed. Proc. **13:** 164, 1954.
38. Winters, R. W., Whitlock, R. T., and Welt, L. G.: The Effect of Dilutional Hyponatremia on Bone Sodium, Clin. Res. **6:** 259, 1958.
39. Levitin, H., Jockers, C. R., and Epstein, F. H.: The Response of Tissue Electrolytes to Respiratory Acidosis, Clin. Res. **6:** 259, 1958.
40. Baum, G., Dick, M., Blum, A., Kaupe, A., and Carballo, J., Jr.: Total Body Exchangeable Potassium and Sodium and Extracellular Fluid in Chronic Pulmonary Insufficiency, Am. Heart J. **58:** 53, 1959.
41. Warner, G. F., Dobson, E. L., Rodgers, C. E., Johnston, M. E., and Pace, N.: The Measurement of Total "Sodium Space" and Total Body Sodium in Normal Individuals and in Patients With Cardiac Edema, Circulation **5:** 915, 1952.
42. Farber, S. J., and Soberman, R. J.: Total Body Water and Total Exchangeable Sodium in Edematous States Due to Cardiac, Renal or Hepatic Disease, J. Clin. Invest. **35:** 779, 1956.
43. Love, W. D., Wild, H. D., and Burch, G. E.: The Potassium, Sodium and Water Content of the Myocardium, Skeletal Muscle, Smooth Muscle and Renal Cortex in Unselected Autopsies, Clin. Res. **6:** 139, 1958.
44. Jaenike, J. R., and Waterhouse, C.: Body Fluid Alterations During the Development of and Recovery From Hyponatremia in Heart Failure, Am. J. Med. **36:** 862, **1959.**
45. Swan, R. C., and Pitts, R. F.: Neutralization of Infused Acid by Nephrectomized Dogs, J. Clin. Invest. **34:** 205, 1955.
46. Schwartz, W. B., Jenson, R. L., and Relman, A. S.: The Disposition of Acid Administered to Sodium-Depleted Subjects: the Renal Response and the Role of the Whole Body Buffers, J. Clin. Invest. **33:** 587, 1954.
47. Pellegrino, E. D., and Farber, S. J.: Human Bone Electrolytes in Various Disease States, Clin. Res. **6:** 259, 1958.
48. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and Le Baron, F. N.: Preparation of Lipide Extracts From Brain Tissue, J. Biol. Chem. **191:** 833, 1951.
49. Forbes, G. B., Tobin, R. B., and Lewis, A.: Response of Bone Sodium to Acute Changes in Extracellular Fluid Composition (Cat), Am. J. Physiol. **196:** 69, 1959.
50. Pellegrino, E. D., and Forbes, S. J.: Human Bone Magnesium, Citrate, and Carbonate. Unpublished data.

ESTIMATION OF HEPATIC BLOOD FLOW IN MAN AND IN DOGS BY I^{131}-LABELED ROSE BENGAL

Simultaneous Comparison With Sulfobromophthalein Sodium

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Hepatic blood flow was estimated simultaneously with I^{131}-labeled rose bengal and sulfobromophthalein sodium in man and in dogs by means of the hepatic clearance-and-extraction method of Bradley. Simultaneous determinations agreed closely over a wide range of values, indicating that I^{131}-labeled rose bengal is suitable for the measurement of hepatic blood flow.

INVESTIGATION of hepatic hemodynamic responses and of hepatic metabolism has been greatly facilitated by the method of Bradley and his associates[2] for the measurement of hepatic blood flow. This procedure is based on the determination of the hepatic clearance and extraction of the dye sulfobromophthalein sodium (BSP). Serious errors are introduced, however, when the plasma is turbid or contains increased amounts of bilirubin or hemoglobin. Under these conditions, the concentration of sulfobromophthalein sodium cannot be accurately determined; it is virtually impossible, therefore, to measure hepatic blood flow in the presence of lipemia, jaundice, or hemolysis.

The dye, rose bengal, may be of value in these situations. This compound has been suggested as a suitable substance for the estimation of hepatic blood flow.[3, 4] When labeled with radioactive iodine, it can be detected readily and with great accuracy regardless of the state of the plasma. Therefore, simultaneous estimations of hepatic blood flow by means of I^{131}-labeled rose bengal and sulfobromophthalein sodium were undertaken in man and in dogs to examine the possibility of using the radioactive compound for hepatic blood flow measurements.

METHODS

Studies were conducted on human subjects and anesthetized dogs (pentobarbital sodium, 30 mg. per kilogram) after an overnight fast. A Cournand needle was placed into a brachial or femoral artery and a hepatic vein was catheterized under fluoroscopic control. A right hepatic vein was uniformly used in man, whereas both right and left hepatic veins were catheterized in dogs. The catheter lumen was kept patent by a slow drip of heparinized saline.

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TABLE I. SIMULTANEOUS COMPARISON OF HEPATIC BLOOD FLOW IN MAN BY I^{131}-LABELED ROSE BENGAL AND SULFOBROMOPHTHALEIN SODIUM

					SULFOBROMOPHTHALEIN SODIUM					I^{131}-LABELED ROSE BENGAL				EHBF BSP / EHBF I^{131} RB	
PATIENT*	AGE	SEX	S.A.† (M^2)	NO. COMPARISONS	PRIME (MG.)	INFUSION RATE (MG./MIN.)	ART. CONC. (MG. %) (RANGE)	EXTRACTION‡ (%) (RANGE)	EHBF (ML./MIN.) (RANGE)	PRIME (μC)	INFUSION RATE (μC/MIN.)	EXTRACTION+ (%) (RANGE)	EHBF (ML./MIN.) (RANGE)	RANGE	AVERAGE
J. C.	38	M	1.74	4	150	3.07	0.39-0.47	55.6-58.3	1,824-2,093	4.0	0.4	25.8-34.3	1,717-2,233	0.94-1.06	1.00
R. W.	24	M	1.53	9§	150	1.45	0.49-0.62	82.0-95.8	883-1,177	5.0	0.5	22.5-29.6	776-1,060	0.91-1.25	1.14
J. F.	55	M	2.17	11§	150	3.71	0.68-0.80	29.5-41.8	1,901-2,535	5.0	0.5	5.6-11.4	1,792-2,546	0.88-1.15	1.02
J. T.	48	M	2.26	3	150	1.88	0.84-0.94	29.6-35.9	937-1,223	4.0	0.4	17.3-19.4	1,010-1,298	0.93-0.97	0.95
A. J.	50	F	1.30	4	150	2.56	1.37-1.52	41.5-52.2	630- 750	4.0	0.4	25.3-30.8	519- 640	0.99-1.36	1.17
C. P.	43	F	1.70	3	150	2.32	0.66-0.72	57.7-60.9	928-1,027	4.8	0.48	19.5-22.9	900-1,120	0.86-1.09	0.99
				3		6.69	1.60-2.12	49.1-62.3	833- 995			12.1-15.2	822- 925	1.01-1.17	1.09
J. G.	37	M	1.87	4	150	3.62	0.82-0.89	64.2-73.4	926-1,140	10.0	0.45	19.2-22.0	1,169-1,261	0.79-0.92	0.86
				3		7.07	2.27-2.52	39.7-45.5	985-1,090			16.5-19.1	1,083-1,226	0.85-0.93	0.89
														Group average = 1.03	

*Diagnoses: J. C., acute gastritis; R. W., diabetes mellitus; J. F., chronic alcoholism; J. T., Laennec's cirrhosis; A. J., carcinoma of pancreatic islet cells; C. P., essential hypertension; J. G., psychoneurosis.

†S.A. = Surface area in square meters.

‡Extraction = $\frac{\text{Arterial concentration} - \text{hepatic venous concentration}}{\text{arterial concentration}} \times 100$.

§Six observations during administration of insulin.

TABLE II. SIMULTANEOUS COMPARISON OF HEPATIC BLOOD FLOW IN DOGS BY I^{131}-LABELED ROSE BENGAL AND SULFOBROMOPHTHALEIN SODIUM

				SULFOBROMOPHTHALEIN SODIUM					I^{131}-LABELED ROSE BENGAL				EHBF BSP / EHBF I^{131} RB	
DOG NO.	WEIGHT (Kg.)	LOBE CATHETERIZED	NO. COMPARISONS	PRIME (MG.)	INFUSION RATE (MG./MIN.)	ART. CONC. (MG. %) (RANGE)	EXTRACTION (%) (RANGE)	EHBF (ML./MIN.) (RANGE)	PRIME (μC)	INFUSION RATE (μC/MIN.)	EXTRACTION (%) (RANGE)	EHBF (ML./MIN.) (RANGE)	RANGE	AVERAGE
1*	18.7	R	8†	100	1.21	0.40-0.48	42.8-52.9	818-1,214	7.0	0.30	16.7-23.9	771-1,328	0.81-1.33	0.97
2	20.0	L	6	75	0.96	0.48-0.68	24.1-30.7	743- 953	2.9	0.32	14.2-21.3	874-1,000	0.77-0.99	0.89
3	34.5	L	9	150	1.16	0.56-0.89	24.6-45.8	591-1,244	2.3	0.25	12.1-23.5	718-1,250	0.79-1.06	0.97
4	30.0	L	12	150	0.90	0.60-0.77	36.5-43.6	458- 611	4.0	0.14	21.1-24.8	378- 603	0.97-1.21	1.08
5	21.3	R	8	100	1.18	0.90-1.30	15.7-27.9	476- 969	2.0	0.25	10.7-17.6	464- 996	0.88-1.05	0.98
6	17.7	R	5	100	1.23	0.91-1.08	18.4-20.4	707- 824	2.0	0.19	18.1-20.6	810-1,030	0.70-0.94	0.79
7	18.1	R	5	80	1.40	1.35-1.62	14.3-16.4	848-1,130	5.0	0.25	11.2-14.4	813- 981	0.86-1.34	1.08
8	15.0	R	4	75	0.90	1.54-1.77	12.5-16.7	415- 671	2.2	0.24	12.0-13.3	454- 540	0.90-1.31	1.07
9	18.9	L	4	60	2.78	2.05-2.40	32.1-45.3	456- 590	5.0	0.5	10.0-14.5	451- 583	0.97-1.02	1.00
10	16.6	L	6	70	2.67	2.30-3.50	32.0-40.9	347- 455	5.0	0.63	8.4-11.8	318- 430	1.00-1.17	1.06
														Group average = 0.995

*Dog 1 femoroportal portacaval shunt; Dog 6 sick animal, bled easily from skin and gums; Dog 8, portacaval shunt.

†Dog 1, 5 observations during insulin infusion; Dog 2, 4 observations after intravenous tolbutamide; Dog 3, 7 observations after intravenous tolbutamide; Dog 4, 9 observations during insulin infusion; Dog 5, 5 observations during insulin infusion.

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Arterial and hepatic venous blood samples were collected simultaneously at 10-minute intervals 20 to 40 minutes after priming doses and constant intravenous infusion of sulfobromophthalein sodium and I^{131}-labeled rose bengal* had been started (quantities administered listed in Tables I and II). Blood samples were placed into tubes which contained powdered heparin and plasma was used for subsequent analyses.

After addition of 0.1N KOH, the optical density due to sulfobromophthalein sodium was determined in duplicate on each plasma sample by reading against a plasma blank diluted to the same extent with alkali in a Beckman DU spectrophotometer set at 575 mμ. The concentration of sulfobromophthalein sodium was determined from a standard curve set up in each study by the addition of known amounts of sulfobromophthalein sodium to the subject's own plasma, which was obtained before dye had been administered.

Plasma radioactivity was measured in duplicate on 1 ml. samples in a deep-well scintillation counter. The coefficient of variation of the mean value of duplicates from a group average was 0.52 per cent over the counting ranges encountered in these studies.

The quantity of sulfobromophthalein sodium and I^{131}-labeled rose bengal administered per minute was calculated from the infusion rate and the respective concentrations of the infused substances.

Hepatic blood flow was estimated for each compound from data interpolated midway between two successive determinations of arterial and hepatic venous sulfobromophthalein sodium concentration and radioactivity by the formula:

$$EHBF = \frac{I \pm \Delta P \bullet PV}{P - H} \times \frac{100}{100 - Het}$$

where EHBF = estimated hepatic blood flow in milliliters per minute.

	BSP	I^{131} RB
I = quantity of infused substance	mg./min.	counts/minute/minute
P = arterial concentration	mg./ml.	counts/minute/ml.
H = hepatic venous concentration	mg./ml.	counts/minute/ml.
ΔP = change in arterial concentration	mg./ml./min.	counts/minute/ml./minute

PV = plasma volume in milliliters calculated from the arterial hematocrit and total blood volume which was estimated to be 80 ml. per kilogram in man and 9 per cent of body weight in the dog. Het = arterial hematocrit.

RESULTS

The data obtained in seven patients and in ten dogs are summarized in Tables I and II, respectively. The range of values has been listed in order to indicate the spread over which comparisons of hepatic blood flow were made.

The ratio $EHBF_{BSP} : EHBF_{I^{131}RB}$ approached unity over a wide range of blood flow values and of arterial sulfobromophthalein sodium concentrations. In man (Table I), the combined average of the ratio $EHBF_{BSP} : EHBF_{I^{131}RB}$ was 1.03 with a range of 0.79 to 1.36 in a series of 44 simultaneous comparisons of hepatic blood flow. In dogs (Table II), the combined average of this ratio in 67 comparisons was 0.995 with a range of 0.70 to 1.34. The relatively wide spread of the ratio $EHBF_{BSP} : EHBF_{I^{131}RB}$ was the consequence of a few divergent results; most of the ratios were close to unity. Thus (Table III), in man 63.6 per cent of the comparisons agreed within ±10 per cent and 81.8 per cent within ±20 per cent. Likewise, in dogs 64.2 per cent of the comparisons were within ±10 per cent and 88.1 per cent within ±20 per cent of unity. Successive values for hepatic blood flow as determined

*I^{131}-labeled rose bengal was purchased from Abbott Laboratories.

by either I^{131}-labeled rose bengal or sulfobromophthalein sodium were observed to vary. Such variation was of the same order of magnitude with either substance, however, since there was no statistically significant difference between the two.

TABLE III. DEVIATION OF INDIVIDUAL COMPARISONS OF SIMULTANEOUS MEASUREMENTS OF HEPATIC BLOOD FLOW BY SULFOBROMOPHTHALEIN SODIUM AND I^{131}-LABELED ROSE BENGAL FROM 100 PER CENT

(%) DEVIATION	HUMAN		DOGS	
	NO. COMPARISONS	%	NO. COMPARISONS	%
±10	28	63.6	43	64.2
±20	36	81.8	59	88.1
±30	43	97.8	64	95.5
Total No. comparisons	44		67	

Where alterations in blood flow were induced, they were detected by both sulfobromophthalein sodium and I^{131}-labeled rose bengal. For example, in Dog 3 (Fig. 1), after two control measurements of hepatic blood flow, 1 Gm. of tolbutamide was administered intravenously over 3 minutes. After this, a progressive fall in arterial glucose concentration ensued. Hepatic blood flow as measured by sulfobromophthalein sodium rose from 817 ml. per minute to a peak of 1,244 ml. per minute and then progressively fell. These changes were followed closely by the I^{131}-labeled rose bengal method.

DISCUSSION

The validity of Bradley's sulfobromophthalein sodium method for measuring hepatic blood flow has been well established.[5, 6] Therefore, sulfobromophthalein sodium was used as the reference substance for comparison of hepatic blood flow values as estimated by I^{131}-labeled rose bengal. Mendeloff,[7] Cohen,[8] and their respective associates have demonstrated that sulfobromophthalein sodium interferes significantly with the hepatic uptake of rose bengal. Rose bengal, in turn, affects uptake of sulfobromophthalein sodium to only a minor extent. These observations do not invalidate measurements of hepatic blood flow when both substances are infused simultaneously, however, provided that the hepatic removal rate and hepatic extraction of both sulfobromophthalein sodium and rose bengal are determined.

Simultaneous measurements of flow by sulfobromophthalein sodium and I^{131}-labeled rose bengal agreed closely over a wide range of values. Agreement was observed not only in humans and dogs with normal hepatic function and an intact splanchnic vasculature, but also in a patient with Laennec's cirrhosis (Patient J. T.), and in dogs with a femoroportal portacaval shunt (Dog 1) and a portocaval shunt (Dog 8). This close agreement persisted even when alterations in hepatic and splanchnic hemodynamics and metabolism were induced by intravenous administration of insulin or tolbutamide. On the basis of these observations it is apparent that I^{131}-labeled rose bengal is a satisfactory substance for the measurement of liver blood flow.

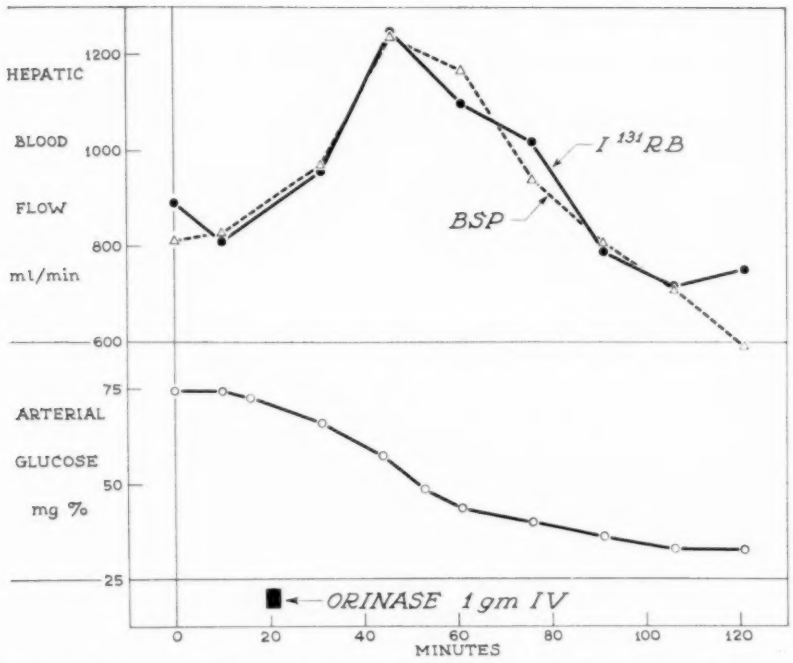

Fig. 1.—Simultaneous measurements of hepatic blood flow by means of I^{131}-labeled rose bengal and sulfobromophthalein sodium were obtained before and after the intravenous administration of 1 Gm. of tolbutamide to Dog 3. In association with the progressive fall of arterial glucose concentration, hepatic blood flow as measured by sulfobromophthalein sodium increased from 817 ml. per minute to a peak of 1,244 ml per minute and then fell progressively. These changes were followed closely by the I^{131}-labeled rose bengal method.

The I^{131}-labeled rose bengal method has distinct advantages. Since radioactivity is measured, pigments and turbidity in plasma do not interfere with accurate determination. It should be possible, therefore, to estimate hepatic blood flow in the presence of lipemia, hemolysis, and jaundice, conditions in which the sulfobromophthalein sodium method cannot be used because of inability to measure the concentration of dye accurately. In addition, the sensitivity of radioactive counting equipment permits the collection of small blood samples for estimation of I^{131} concentration. This is particularly valuable in studies when frequent measurements of hepatic blood flow over a prolonged period are desired. Thus, one half to one third as much blood is required for measurement of hepatic blood flow by I^{131}-labeled rose bengal as with sulfobromophthalein sodium. In recent studies,[9] the I^{131}-labeled rose bengal method has been simplified further. The blood samples are collected in heparinized tubes and then hemolyzed with saponin. Radioactivity of the hemolyzate is measured and hepatic blood flow is calculated directly from these data.

The quantity of radioactivity that need be infused in man is not excessive. A total dose of less than 100 μc would be administered if a 5 μc prime and a constant infusion of 0.5 μc per minute were administered over a period of 3 hours, during which time repeated measurements of flow could be obtained.

SUMMARY

Simultaneous measurements of hepatic blood flow were made in man and in dogs by the hepatic clearance-and-extraction method using sulfobromophthalein sodium and I^{131}-labeled rose bengal. The combined averages of the ratio, estimated hepatic blood flow as measured by sulfobromophthalein sodium to estimated hepatic blood flow as measured by I^{131}-labeled rose bengal, under a variety of circumstances were 1.03 for 44 comparisons in man and 0.995 for 67 comparisons in dogs. Such data indicate that I^{131}-labeled rose bengal is a satisfactory substance for the determination of liver blood flow.

I am indebted to Dr. Mitchell Young, Mrs. Mary Hamilton, and Mrs. Ruthie Rose for their valuable technical assistance.

REFERENCES

1. Combes, B.: Simultaneous Estimation of Hepatic Blood Flow in Dogs by Means of I^{131}-Labeled Rose Bengal (I^{131} RB) and Bromsulfalein (BSP), Circulation **18**: 705, 1958. (Abst.)
2. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J.: The Estimation of Hepatic Blood Flow in Man, J. Clin. Invest. **24**: 890, 1945.
3. Sapirstein, L. A., and Simpson, A. M.: Plasma Clearance of Rose Bengal (Tetraiodotetrabromfluorescein), Am. J. Physiol. **182**: 337, 1955.
4. Combes, B., Wheeler, H. O., Childs, A. W., and Wade, O. L.: The Effect of 2,4-Dinitrophenol on the Hepatic Clearance and Extraction of Rose Bengal in Dogs, Fed. Proc. **15**: 38, 1956. (Abst.)
5. Selkurt, E. E.: Validity of the Bromsulphalein (BSP) Method for Estimating Hepatic Blood Flow, Am. J. Physiol. **175**: 461, 1953.
6. Selkurt, E. E.: Comparison of the Bromsulphalein Method With Simultaneous Direct Hepatic Blood Flow, Circulation Res. **2**: 155, 1954.
7. Mendeloff, A. I., Kramer, P., Ingelfinger, F. J., and Bradley, S. E.: Studies With Bromsulfalein. II. Factors Altering Its Disappearance From the Blood After a Single Intravenous Injection, Gastroenterology **13**: 222, 1949.
8. Cohen, E. S., Giansiracusa, J. E., and Althausen, T. L.: Studies on Bromsulfalein Excretion. III. The Simultaneous Performance of the Bromsulfalein and Rose Bengal Excretion Tests in Individuals With Normal Hepatic Function, Gastroenterology **25**: 237, 1953.
9. Madison, L. L., Combes, B., Adams, R., and Strickland, W.: The Physiological Significance of the Secretion of Endogenous Insulin Into the Portal Circulation. III. Evidence for a Direct Immediate Effect of Insulin on the Balance of Glucose Across the Liver, J. Clin. Invest. **39**: 507, 1960.

SERUM HEXOSAMINE CONTENT AND URINARY ACID MUCOPOLYSACCHARIDE EXCRETION IN HEREDITARY PRIMARY AMYLOIDOSIS

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Serum hexosamine levels by the Elson-Morgan technique were found to be normal in eleven patients from a previously reported family with hereditary primary amyloidosis. Urinary acid mucopolysaccharide excretion measured by the method of DiFerranti and Rich in eight of these patients was also normal. Paper electrophoretic strips stained by the modified Schiff reaction revealed no consistent difference between the pattern of normal and amyloid serum.

ONE characteristic of amyloidosis is deposition in the body of amyloid, believed to be chiefly a mucoprotein. Hydrolysis of the mucopolysaccharide moiety of this mucoprotein yields the hexosamine, glucosamine,[1] which Faber[2] and Calkins and Cohen,[3] have reported to be elevated in the serum in a study of a small series of patients with amyloidosis. Giles and Calkins[4] have also reported elevation of serum hexosamine in experimental amyloidosis. The presence of an atypical protein peak between alpha$_2$ and beta globulin fractions when serum from patients with hereditary primary amyloidosis was subjected to electrophoresis was reported by Rukavina and co-workers.[5] Some mucoproteins are known to migrate in the same electrophoretic area as the observed atypical peak at pH of 8.6.

The finding of abnormal levels of serum glucosamine or increased urinary excretion of mucopolysaccharides would be extremely important in the clinical diagnosis of this disease. Therefore, these compounds were determined in a series of patients with hereditary primary amyloidosis who were members of a previously reported large family with amyloidosis.[6]

MATERIALS AND METHODS

Fasting blood specimens were obtained from normal subjects and from 11 patients with proved amyloidosis or amyloidosis suspected on the basis of clinical findings and relationship to patients with the disease. Three consecutive 24 hour urine specimens were also collected from 8 of these patients. Urine creatinine determinations were made as a check of the accuracy of collections.

Serum hexosamine content was determined by the method of Elson and Morgan.[7] The serum was subjected to paper strip electrophoresis by the method of Block and collaborators[8] at a pH of 8.6, and the paper strips were stained with a modified Schiff

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reagent.[9] Free electrophoretic studies of the sera at a pH of 4.5 were performed to determine the amount of mucoprotein present by the technique of Mehl and associates.[10] Acid mucopolysaccharide excretion was determined by the method of DiFerranti and Rich.[11]

RESULTS

Serum.—Serum hexosamine content in both the normal subjects and the patients with hereditary primary amyloidosis is given in Table I. Values for the control subjects ranged from 68.2 to 114.5 mg. per cent with an average of 91.3 mg. per cent; those for the patients with amyloidosis ranged from 83.0 to 128.1 mg. per cent with an average of 102.7 mg. per cent. The paper electrophoretic strips stained by the modified Schiff reaction revealed no consistent abnormality. At a pH of 4.5 free electrophoresis of serum from the patients for mucoprotein showed prealbumin peaks similar in amount to those obtained with normal serum.

TABLE I. SERUM HEXOSAMINE CONTENT AND URINARY ACID MUCOPOLYSACCHARIDE EXCRETION IN HEREDITARY PRIMARY SYSTEMIC AMYLOIDOSIS

SERUM HEXOSAMINE CONTENT (MG. %)		ACID MUCOPOLYSACCHARIDE EXCRETION (MG./24 HR.)	
CONTROL	AMYLOIDOSIS	CONTROL	AMYLOIDOSIS
68.2	83.0	2.08	2.08
81.9	87.5	2.83	2.76
82.2	91.0	2.92	3.25*
84.2	92.2	3.50	3.52
89.0	101.3	3.72	3.80*
90.9	103.8	3.83	4.30
97.8	104.1*	4.67	6.82
99.9	110.4	5.06	8.41
104.2	111.9*	8.44	
114.5	115.9		
	128.1*		
Average 91.3	102.7	4.12	4.37
Range 68.2-114.5	83.0-128.1	2.08-8.44	2.08-8.41

*Diagnosis of amyloidosis confirmed by pathologic findings in these patients.

Urine.—Excretion of acid mucopolysaccharide by the control subjects ranged from 2.08 to 8.44 mg. per 24 hours with an average of 4.12 mg.; those for the patients with hereditary primary amyloidosis ranged from 2.08 to 8.41 mg. per 24 hours with an average of 4.37 mg. (Table I).

DISCUSSION

Serum glucosamine values have been reported to be elevated in amyloidosis,[2, 3] but the data from the present study indicate that in hereditary primary systemic amyloidosis no such elevation occurs. Calkins and Cohen[3] found elevation of serum hexosamine above 120 mg. per cent in eight of nine patients with primary amyloidosis. The ninth patient without elevation was thought to have the familial variety of primary amyloidosis. These findings might suggest that there is a difference in serum hexosamine level between patients with the hereditary type and those with the nonhereditary type of primary amyloidosis. This possibility needs further elucidation.

The finding of normal acid mucopolysaccaride excretion in these patients does not exclude the possibility that some specific mucopolysaccharides might be excreted in abnormal amounts in hereditary primary systemic amyloidosis. This possibility is being studied further.

SUMMARY

Patients with hereditary primary systemic amyloidosis had normal levels of serum glucosamine and urinary acid mucopolysaccharide excretion. Electrophoretic strips stained by the modified Schiff reaction revealed no consistent difference between the pattern of the sera from patients with amyloidosis and normal subjects.

REFERENCES

1. Thompson, R. H. S., and King, R. J.: Biochemical Disorders in Human Disease, New York, 1957, Academic Press, Inc., pp. 648 and 649.
2. Faber, M.: Serum Glucosamine With Particular Regard to Its Significance in Connection With the Origin of Amyloid Deposits, Acta med. scandinav., Supp. **206**: 351, 1948.
3. Calkins, E., and Cohen, A. S.: Similarity of Serum Protein Changes in Primary and Secondary Amyloidosis, J. Clin. Invest. **38**: 993, 1959.
4. Giles, R. B., Jr., and Calkins, C.: The Relationship of Serum Hexosamine, Globulins, and Antibodies to Experimental Amyloidasis, J. Clin. Invest. **37**: 846, 1958.
5. Rukavina, J. G., Block, W. D., and Curtis, A. C.: Familial Primary Systemic Amyloidosis: an Experimental, Genetic and Clinical Study, J. Invest. Dermatol. **27**: 111, 1956.
6. Rukavina, J. G., Block, W. D., Jackson, C. E., Falls, H. D., Carey, J. H., and Curtis, A. C.: Primary Systemic Amyloidosis: A review and an Experimental, Genetic, and Clinical Study of 29 Cases With Particular Emphasis on the Familial Form, Medicine **35**: 239, 1956.
7. Elson, L. A., and Morgan, W. T. J.: A Colorimetric Method for the Determination of Glucosamine and Chondrosamine, Biochem. J. **27**: 1824, 1933.
8. Block, R. J., Durram, E. L., and Zweig, G.: A Manual of Paper Chromatography and Paper Electrophoresis, ed. 2, Part II, New York, 1958, Academic Press, Inc.
9. Shetlar, M. R., Cahill, C., Stidworthy, G., and Shetlar, C. L.: Comparison of Continuous and Paper Strip Electrophoresis Techniques for Study of Serum Glycoproteins, Proc. Soc. Exper. Biol. & Med. **93**: 44, 1956.
10. Mehl, J. W., Golden, F., and Winzler, R. J.: Mucoproteins of Human Plasma. IV. Electrophoretic Demonstration of Mucoproteins in Serum at pH 4.5, Proc. Soc. Exper. Biol. & Med. **72**: 110, 1949.
11. DiFerrante, N., and Rich, C.: The Determination of Acid Aminopolysaccharide in Urine, J. Lab. & Clin. Med. **48**: 491, 1956.

DISTRIBUTION OF RADIOACTIVITY IN MAN AFTER THE ORAL INGESTION OF SMALL TEST DOSES OF RADIOLABELED CYANOCOBALAMIN

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This study was made to determine the distribution of orally ingested doses of radiolabeled cyanocobalamin of physiologic size in the presence of intrinsic factor. Test doses were administered to seriously ill patients and, in eleven subjects who died 2 hours to 113 days later, the radioactivity of various organs was measured. There was a temporary storage of vitamin B_{12} in the wall of the small intestine. The most rapid transfer of the vitamin to other organs occurred between hours 7 and 24 with a more gradual transfer subsequently. In the majority of the patients the ileum was the most important site for the temporary storage of radioactivity and presumably for the absorption of cyanocobalamin. The liver, spleen, and kidneys had approximately the same concentrations of radioactivity during the first 2 days, then the concentration increased in the liver while it decreased in the spleen and the kidneys. Absorption of vitamin B_{12} was impaired shortly before death even in the presence of adequate amounts of intrinsic factor.

PLASMA radioactivity curves made after the oral administration of small test doses of radiolabeled cyanocobalamin have shown a unique pattern characterized by a delay of 3 to 4 hours before the appearance of significant radioactivity with peak concentrations in the 8 to 12 hour interval.[1-4] Subsequent absorption studies on blood from the portal vein, in patients with portacaval shunts and in patients who had undergone ileostomies and total colectomies, have indicated that the wall of the small bowel was responsible for the delayed appearance of radioactivity in the plasma.[5] These observations led to the conclusion that the wall of the small intestine serves as a temporary storage organ for cyanocobalamin during its absorption from the gastrointestinal tract.[5] Since this conclusion was drawn from indirect evidence the purpose of the present study was to make direct observations on the distribution of radioactivity at differing time intervals after the oral administration of small doses of radiolabeled cyanocabalamin in the presence of intrinsic factor. The test doses

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TABLE I. DISTRIBUTION OF RADIOACTIVITY IN VARIOUS ORGANS AFTER ORAL INGESTION OF RADIOLABELED CYANOCOBALAMIN (0.56 MCG. VITAMIN B_{12} CONTAINING 0.5 μC Co^{60})

SUBJECT NO.	AGE	DIAGNOSIS	TIME INTERVAL BETWEEN TEST AND DEATH (HR.)	ORAL DOSE (C.P.M.)	ORGAN RADIOACTIVITY PER CENT OF ORAL DOSE				$\left(\frac{A}{A+B}\right)100$
					A SMALL INTESTINE	B EXTRA-INTESTINAL ORGANS*	C SKELETAL MUSCULATURE†	D SKELETON†	
1	34	Hodgkin's disease	2	252,902	1.3	0.1‡	0	-	91.6
2	67	Myocardial infarction	4	248,853	13.4	0.5‡	5.2‡	1.3‡	95.9
3	70	Emphysema pneumonia	7	235,194	15.2	0.5‡	0.7‡	0.4‡	96.9
4	63	Myocardial infarction	24	237,114	1.5	3.4	2.0‡	2.3‡	30.2
5	27	Cancer of stomach	42	252,221	3.6	9.6	4.3	3.4	27.5
6	64	Arteriosclerotic heart disease	85	248,833	0.7	10.3	0	1.2‡	6.4
7	70	Bronchial carcinoma	115	237,497	2.5	48.1	3.1‡	3.1	4.9
8	85	Cerebral thrombosis	137	253,124	3.0	20.2	6.3	2.2	12.9
9	63	Arteriosclerotic heart disease	309	235,325	2.5	26.2	7.1	1.7	8.6
10	64	Hodgkin's disease	724	247,736	0.4	29.9	2.8‡	1.2‡	1.4
11	68	Cerebral thrombosis	2,724	253,538	0.5	30.9	11.8	2.4‡	1.5

*Includes liver, spleen, kidneys, pancreas, lungs, and heart.
†Estimated weights.[7]
‡Statistically not significant.

TABLE II. DISTRIBUTION OF RADIOACTIVITY IN EXTRAINTESTINAL ORGANS

SUBJECT	HOURS	LIVER			SPLEEN			KIDNEYS			PANCREAS			LUNGS			HEART		
		A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
4	24	74.9	337	26.9	6.8	427	34.1	9.1	266	21.2	1.4	129	10.3	5.7	61	4.9	2.1	34	2.7
5	42	78.3	750	32.6	5.1	448	19.5	14.6	883	38.4	0.6	133	5.8	--	--	--	1.3	84	3.7
6	85	90.1	1,515	63.0	2.6	369	15.3	2.1	231	9.6	0.4	175	7.3	2.6	45	1.9	1.3	69	2.9
7	115	90.7	7,058	68.0	1.4	1,116	10.7	2.2	746	7.2	0.9	988	9.5	4.3	270	2.6	0.5	206	2.0
8	137	83.7	4,166	65.2	1.7	829	13.0	2.4	316	4.9	0.3	474	7.4	10.8	433	6.8	1.1	172	2.7
9	309	88.7	4,291	60.8	0.5	515	7.3	4.4	872	12.4	1.1	870	12.3	3.7	289	4.1	1.4	215	3.0
10	424	89.1	4,519	54.6	1.5	435	5.3	6.3	2,519	30.4	0.4	471	5.7	1.9	156	1.9	0.8	179	2.2
11	2,724	92.6	4,841	66.3	0.4	287	3.9	2.4	615	8.4	1.0	933	12.8	1.9	116	1.6	1.8	511	7.0

A = percentage of total extraintestinal radioactivity.
B = observed counts per minute per 100 grams of tissue.
C = percentage of total radioactivity per 100 grams of tissue.

were administered to seriously ill patients and, whenever postmortem examinations were obtained, the distribution of radioactivity in the gastrointestinal tract and other organs was measured by direct counting. Particular attention was directed to details of the distribution of radioactivity in the small intestine. Results have been obtained in eleven subjects who died from 2 hours to 113 days after the ingestion of the radiolabeled vitamin.

MATERIALS AND METHODS

An oral test dose of 0.56 mcg. of cyanocobalamin which contained 0.5 μc of Co^{60}* was administered in 20 ml. of water after an overnight fast in five patients and between breakfast and noon in six. Because the status of intrinsic factor production was unknown in the subjects, one standard unit of intrinsic factor concentrate (IFC)† was always added to the labeled cyanocobalamin immediately before the tests. The radioactivity was determined on weighed samples of liver, spleen, kidneys, pancreas, lungs, heart muscle, pectoralis muscle, and rib. The wet tissue samples were placed in 20 ml. disposable vials and counted in a well-type scintillation counter[6] employing a preset count of 12,800. Seven counts per minute exceeded 3 times the standard deviation of the background and, therefore, were considered significant. Total organ radioactivity was calculated from the activity of the samples and the wet weights of the respective organs. In the case of the skeleton and skeletal musculature, however, the activity was calculated from their estimated weights, namely 13.2 per cent of the body weight for the skeleton and 39.7 per cent for the musculature.[7] The gastrointestinal tract was rinsed with running tap water and, starting from the pylorus, the small intestine was cut into 1 foot segments which were counted individually. The total radioactivity of the small intestine was the sum of the counts of these segments. In some instances the activities of the stomach and 1 foot segments of ascending, transverse, and sigmoid colon, respectively, were obtained in a similar manner.

RESULTS

Table I summarizes the results of the studies. Comparison of the radioactivity of the small intestine with the total visceral radioactivity shows that in the three patients who died during the first 7 hours after the tests, virtually all of the visceral counts were found in the wall of the small bowel. However, by 24 hours the radioactivity of the small intestine had dropped to 30 per cent and by 42 hours to 28 per cent of the total visceral counts. A further decline to between 5 and 13 per cent was recorded 85 to 309 hours after the tests, and to between 1 and 2 per cent in the two subjects who died 724 and 2,724 hours after ingestion of the vitamin.

In the 6 subjects who died during the first 85 hours after the tests the total organ radioactivity ranged from 1.4 to 20.9 per cent of the ingested dose as compared to 31.7 to 56.8 per cent in those who died later. These figures indicated an impaired absorption of the vitamin shortly before death.

Details of the distribution of radioactivity in the small intestine are shown in Fig. 1. For comparison, in each case the organ was divided into 10 equal parts and the radioactivities of the corresponding 1 foot segments were assigned appropriately. The radioactivity of each tenth was then expressed as per cent of its respective whole. The radioactivity was usually distributed unevenly.

*Co^{60}-labeled vitamin B_{12} supplied by Dr. N. S. Ritter, Merck & Co., Inc., Rahway, N. J.
†Intrinsic factor concentrate standard supplied by Dr. Paul J. Fouts, Eli Lilly and Company, Indianapolis, Ind.

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The ileum showed preferential storage of radioactivity in seven of the subjects, the jejunum in two, whereas an even or almost even distribution was recorded in the last two patients in the series.

Table II summarizes the distribution of radioactivity in the liver, spleen, kidneys, pancreas, lungs, and heart of the last eight subjects. The calculated total organ radioactivity (A) as well as the concentration of radioactivity in each organ (B and C) has been considered. The first three subjects in our series are not included because, as indicated in Table I, the measured radioactivity of their extragastrointestinal organs was too negligible for reliable calculations. Comparison of lines A shows that the liver contained from 75 to 93 per cent of all the extraintestinal activity and was, therefore, the most important single organ for the permanent storage of cyanocobalamin.

The radioactivity of the extraintestinal organs was also compared on the basis of concentration. Because of the great individual differences in the total amounts of radioactivity observed (lines B), the comparison was made easier by using the relative concentration figures (lines C). For the spleen and the kidneys these figures were at their maximum at 24 and 42 hours and they did not differ materially from those of the liver. The values for the spleen and the kidneys subsequently decreased, whereas those for the liver increased.

The amounts of radioactivity found in the musculature and the skeleton, Table I, and in the stomach and colon, Table III, were rather small and inconsequential.

TABLE III. RADIOACTIVITY OF STOMACH AND 1 FOOT SEGMENTS OF ASCENDING, TRANSVERSE, AND SIGMOID COLON

SUBJECT NO.	TIME INTERVAL BETWEEN TEST AND DEATH (HR.)	ORAL DOSE (C.P.M.)	C.P.M. REGAINED			
				COLON		
			STOMACH	ASCENDING	TRANSVERSE	SIGMOID
2	4	248,853	141			
3	7	235,194	533	46	28	51
4	24	237,114	154	31	44	21
6	85	248,833	65			
7	115	237,497	246	83	54	76
9	309	235,325	346	53	39	42
10	724	247,736	138			
11	2,724	253,538	174			

DISCUSSION

Because these studies were carried out on seriously ill patients, there may be objections to generalizations from the results. However, in these patients, the wall of the small intestine served as a temporary storage organ for the radiocyanocobalamin. There was a delayed release and subsequent gradual transfer of the vitamin to other viscera, mainly the liver. These findings confirmed inferences and conclusions drawn from less direct evidence by ourselves and others.[5, 8]

The total visceral radioactivity was lower in the subjects who died during the first 3½ days after the tests than in those who died later. In other words, shortly before death the absorption of cyanocobalamin was diminished even in

the presence of adequate amounts of intrinsic factor. It can only be speculated as to the cause of the impaired absorption. While infections may be responsible for a decreased absorption of vitamin B_{12},[9, 10] this could have been a possible explanation in only one subject (Subject 3, Table I).

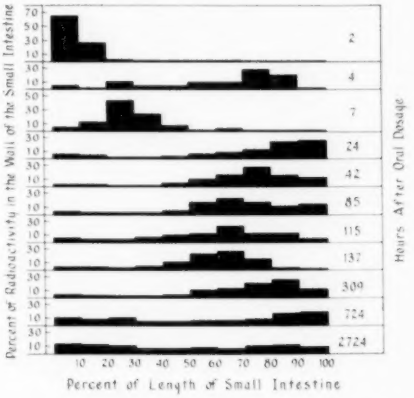

Fig. 1.—Distribution of radioactivity in the wall of the small intestine at various time intervals after the oral administration of 0.56 mcg. cyanocobalamin containing 0.5 μc Co^{60}

Although large doses of intrinsic factor concentrate can lower the absorption of cyanocobalamin in man,[11, 12] significant inhibition was not noted until the optimum of intrinsic factor concentrate had been exceeded many times.[12] Our subjects did not receive excessive amounts of this substance and, furthermore, they all received the same dose of intrinsic factor concentrate. Therefore, it is unlikely that the addition of this factor caused the lowered absorption.

Since the gastrointestinal assimilation of small amounts of cyanocobalamin is probably an active process of absorption, it is possible that the impairment of this function shortly before death was related to a general deterioration of all the vital processes in these seriously ill patients.

An uneven distribution of radioactivity was found in the small intestine in most of the subjects. This finding may have an important bearing upon the controversial question as to the site of absorption of cyanocobalamin. Some reports favor absorption from the upper,[13, 14] some from the lower small intestine,[15-20] and one report suggests that the whole length of the small bowel is involved[21] as found in the rat.[22, 23] The categorical denial of absorption from the upper small intestine[20] is contradicted by individual instances with normal absorption from the jejunum[14] or the upper half of the small intestine.[16]

If it is assumed that the vitamin is absorbed from the area of preferential binding, in our subjects the ileum was the most important site for absorption. On the other hand, in each of the 2 patients who died at 2 and 7 hours, respectively, there was more radioactivity in the jejunum. However, the significance of these two observations with respect to absorption is open to further interpretation. For example, negligible radioactivity was found in the extraintestinal organs, but this may have been due to the fact that little absorption of cyanocobalamin normally takes place during the first 4 to 6 hours.[24] The preferential

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binding of radioactivity in the jejunum in the patient who died after 2 hours may have been due to insufficient time for distribution throughout the intestine. On the other hand, this possibility was less likely in the case of the patient who died after 7 hours because, for comparison, the subject who died at 4 hours already had increased amounts of radioactivity in the wall of the ileum. The finding of preferential binding in the jejunum, especially in the patient who died at 7 hours, suggests that the jejunum cannot be excluded as an area of absorption at least in some patients.

The even or almost even distribution of radioactivity along the entire length of the small intestine in the last 2 subjects who died at 30 and 113 days, respectively, could possibly mean an even absorption from the small intestine. However, since the small bowel contained only 0.4 and 0.5 per cent of the ingested radioactivity at this time, it is more likely that the residual activity represented either permanent storage or utilization of cyanocobalamin in the bowel wall.

The finding that the liver was the most important organ for the permanent storage of cyanocobalamin is consistent with previous observations of in vivo radioactivity measurements[25-27] as well as with the results of bioassays for the cyanocobalamin activity of various organs.[28]

Whereas an almost equal concentration of radioactivity was found in the liver, spleen, and kidneys 24 and 42 hours after the tests, in the 6 subjects who died later the concentration in the liver was 2 to 17 times higher than that in the spleen or the kidneys. Although more data are needed to be certain, it appears from these few observations that the spleen and the kidneys may play a more important role in the storage of cyanocobalamin shortly after absorption than later. Such a finding suggests that newly absorbed vitamin B_{12} is shifted around between the extraintestinal organs during the first few days after the tests. Observations made after parenteral injections of labeled cyanocobalamin in man[26, 29] and in rats after parenteral[30] and oral[22] administration may be interpreted in a similar manner. In short, the spleen and the kidneys may serve as an extraintestinal temporary storage site for cyanocobalamin.

The wall of the stomach and colon contained inconsequential amounts of radioactivity. This finding is in line with previous reports that vitamin B_{12} in physiologic amounts is not absorbed from the colon[21] and that the stomach inhibits the absorption of this vitamin.[21, 31, 32] The amounts of radioactivity in the skeleton and the skeletal musculature must be taken as only rough estimates since the true weights of these organs were unknown. Furthermore, samples of these structures might not have been representative. Nevertheless, there were small quantities of radioactivity present in both musculature and bones. These findings are consistent with the observations recorded in man after parenteral administration of radiolabeled cyanocobalamin.[27]

SUMMARY

1. Physiologic test doses of radiolabeled cyanocobalamin were administered to eleven seriously ill patients who died from 2 hours to 113 days later. The

distribution of radioactivity in various organs was determined directly in tissues obtained at autopsy.

2. In 3 patients who died within 7 hours of the tests, practically all of the visceral radioactivity was found in the wall of the small intestine. This indicated temporary storage of vitamin B_{12} in the intestinal wall. The rate of transfer of radioactivity to the permanent storage organs was greatest between 7 and 24 hours and continued thereafter in a declining manner for several days.

3. The radioactivity was not evenly distributed along the entire length of the small intestine. Usually the ileum was the most important site for the temporary storage of radioactivity and presumably for the absorption of cyanocobalamin. However, in two instances the jejunum showed preferential binding of the vitamin.

4. The liver was the most important organ for the permanent storage of the radioactivity.

5. The spleen and the kidneys had higher concentrations of the labeled cyanocobalamin during the first 2 days after the tests than subsequently.

6. The absorption of vitamin B_{12} was impaired shortly before death even in the presence of adequate amounts of intrinsic factor.

REFERENCES

1. Booth, C. C., and Mollin, D. L.: Plasma, Tissue and Urinary Radioactivity After Oral Administration of Co^{56}-Labelled Vitamin B_{12}, Brit. J. Haemat. **2**: 223, 1956.
2. Doscherholmen, A., and Hagen, P. S.: Radioactive Vitamin B_{12} Absorption Studies: Results of Direct Measurement of Radioactivity in the Blood, Blood **12**: 336, 1957.
3. Doscherholmen, A., and Hagen, P. S.: A Dual Mechanism of Vitamin B_{12} Plasma Absorption, J. Clin. Invest. **36**: 1551, 1957.
4. Goldberg, S. R., Trivedi, B. K., and Oliner, L.: Radioactive Vitamin B_{12} Studies. Experience With the Urinary Excretion Test and the Measurement of Absorbed Plasma Radioactivity, J. LAB. & CLIN. MED. **49**: 583, 1957.
5. Doscherholmen, A., and Hagen, P. S.: Delay of Absorption of Radiolabeled Cyanocobalamin in the Intestinal Wall in the Presence of Intrinsic Factor, J. LAB. & CLIN. MED. **54**: 434, 1959.
6. Baskin, R., Demorest, H. L., and Sandhaus, S.: Gamma Counting Efficiency of Two Well-type NaI Crystals, Nucleonics **12**: 46, August, 1954.
7. Schaeffer, J. P.: Morris' Human Anatomy, New York, 1953, Blakiston Company, p. 43.
8. Glass, G. B. J., and Schaeffer, H.: Further Observations on the Intestinal Absorption of Vitamin B_{12} as Measured by Hepatic Uptake of $Co^{60}B_{12}$, Bull. New York M. Coll. **19**: 74, 1956.
9. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H.: Studies of Excretion (and Absorption) of Cobalt^{60} Labelled Vitamin B_{12} in Pernicious Anemia, Tr. A. Am. Physicians **65**: 214, 1952.
10. Callender, S. T., Turnbull, A., and Wakisaka, G.: Estimation of Intrinsic Factor of Castle by Use of Radioactive Vitamin B_{12}, Brit. M. J. **1**: 10, 1954.
11. Glass, G. B. J., Boyd, L. J., Stephanson, L., and Jones, E. L.: Metabolic Interrelations Between Intrinsic Factor and Vitamin B_{12}. III. B_{12} Absorption at Varied Intrinsic Factor Doses, Proc. Soc. Exper. Biol. & Med. **88**: 1, 1955.
12. Callender, S. T., and Evans, J. R.: Observations on the Relationship of Intrinsic Factor to the Absorption of Labelled Vitamin B_{12} from the Intestine, Clin. Sc. **14**: 387, 1955.
13. Castle, W. B.: Development of Knowledge Concerning the Gastric Intrinsic Factor and Its Relation to Pernicious Anemia, New England J. Med. **249**: 603, 1953.
14. Quinby, W. C., and McGovern, J. J.: Surgical Correction of Defective Absorption of Vitamin B_{12} in a Child, New England J. Med. **259**: 755, 1958.
15. McIntyre, P. A., Sachs, M. V., Krevans, J. R., and Conley, C. L.: Pathogenesis and Treatment of Macrocytic Anemia; Information Obtained With Radioactive Vitamin B_{12}, A. M. A. Arch. Int. Med. **98**: 541, 1956.

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16. Kogan, E., Schapira, A., Janowitz, H. D., and Aldersberg, D.: Malabsorption Following Extensive Small Intestinal Resection Including Inadvertent Gastro-ileostomy, J. Mt. Sinai Hosp. New York **24**: 399, 1957.
17. Cooke, W. T., Cox, E. V., Meynell, M. J., and Gaddie, R.: Importance of the Ileum in the Absorption of Vitamin B_{12}, Lancet **2**: 1231, 1957.
18. Gardner, F. H.: Gastrointestinal Flora and the Red Cells, Am. J. Digest Dis. **2**: 175, 1957.
19. Booth, C. C., and Mollin, D. L.: The Site of Absorption of Vitamin B_{12} in Man, Lancet **1**: 18, 1959.
20. Best, W. R., Frenster, J. H., and Zolot, M. M.: Observations on Intrinsic Factor and the Intestinal Absorption of Vitamin B_{12}, J. LAB. & CLIN. MED. **50**: 793, 1957. (Abst.)
21. Citrin, Y., DeRosa, C., and Halsted, J. A.: Sites of Absorption of Vitamin B_{12}, J. LAB. & CLIN. MED. **50**: 667, 1957.
22. Booth, C. C., Chanarin, I., Anderson, B. B., and Mollin, D. L.: The Site of Absorption and Tissue Distribution of Orally Administered Co^{56}-Labelled Vitamin B_{12} in the Rat, Brit. J. Haemat. **3**: 253, 1957.
23. Reynell, P. C., Spray, G. H., and Taylor, K. B.: The Site of Absorption of Vitamin B_{12} in the Rat, Clin. Sc. **16**: 663, 1957.
24. Doscherholmen, A., and Hagen, P. S.: Kinetic Aspects of Gastrointestinal Absorption of Cyanocobalamin, Fortieth Annual Session, The American College of Physicians, April 20-24, 1959, Chicago, Ill. To be published.
25. Glass, G. B. J., Boyd, L. J., Gellin, G. A., and Stephanson, L.: Uptake of Radioactive Vitamin B_{12} by the Liver in Humans: Test for Measurement of Intestinal Absorption of Vitamin B_{12} and Intrinsic Factor Activity, Arch. Biochem. **51**: 251, 1954.
26. Glass, G. B. J., Boyd, L. J., and Gellin, G. A.: Surface Scintillation Measurements in Humans of the Uptake of Parenterally Administered Radioactive Vitamin B_{12}, Blood **10**: 95, 1955.
27. Cronkite, E. P., Henley, E., Driscoll, D. L., Meyer, L. M., Dohan, F. C., Rubini, J. R., and Wollins, W.: Studies on the Kinetics of Intravenously Injected $Cobalt^{60}$ Labeled Vitamin B_{12} in Man, Presented at the Seventh Congress of the International Society of Hematology, Rome, Italy, September, 1958.
28. Halsted, J. A., Carroll, J., and Rubert, S.: Serum and Tissue Concentration of Vitamin B_{12} in Certain Pathologic States, New England J. Med. **260**: 575, 1959.
29. Meyer, L. M., Berlin, N. I., Jiminez-Casado, M., and Arkun, S. N.: Vitamin B_{12} Distribution, Determined by Surface Body Counting Following Parenteral Administration of Co^{60} Vitamin B_{12}, Proc. Soc. Exper. Biol. & Med. **91**: 129, 1956.
30. Harte, R. A., Chow, B. F., and Barrows, L.: Storage and Elimination of Vitamin B_{12} in the Rat, J. Nutrition **49**: 669, 1953.
31. Halsted, J. A., Gasster, M., and Drenick, E. J.: Absorption of Radioactive Vitamin B_{12} After Total Gastrectomy, New England J. Med. **251**: 161, 1954.
32. Johnson, P. C., and Berger, E. S.: Enhanced Urinary Excretion of Co^{60} Vitamin B_{12} Produced by Delayed Release Capsules, Blood **13**: 457, 1958.

LOWERING OF TRANSFERRIN DURING IRON ABSORPTION IN IRON DEFICIENCY

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A substantial dose of ferrous sulfate given orally in the fasting state to iron-deficient subjects was followed by a lowering of the total iron binding capacity of the serum in 22 of 32 subjects. Return of the total iron binding capacity toward normal was observed after 6 to 8 hours. The serum iron concentration was elevated. In 12 subjects the lowering of the total iron binding capacity during iron absorption was associated with complete saturation of the circulating transferrin. The results suggest the presence of mechanisms which cause sudden changes in the concentration of circulating transferrin.

An explanatory hypothesis of the results is presented. The hypothesis considers transferrin to exist in two phases: one circulating in plasma and the other sequestered at cellular sites. It is proposed that sequestration may result from orientation of iron laden transferrin to iron receptors at cellular sites during the transferral of the iron to cells. Thus, in iron deficiency the circulating transferrin may be increased at the expense of the sequestered portion, whereas in hemochromatosis the reverse may pertain.

THREE terms are used in describing the iron transport system.[1] These terms, expressed as micrograms of iron per 100 ml. are: the plasma or serum iron concentration (PlFe or SeFe), the total iron binding capacity (TIBC), and the unsaturated iron binding capacity (UIBC). At present it is common practice to measure SeFe and UIBC, and to add these two quantities in order to obtain the TIBC[1, 2] which becomes an expression of the concentration of transferrin.

In iron-deficient and pregnant individuals the SeFe is lowered and the TIBC is elevated; in hemochromatosis, pernicious anemia in relapse, and chronic hemolytic states, the SeFe is elevated and the TIBC remains unchanged or is lowered. No conceptual interpretation of these variations in SeFe and TIBC has been established. The problem of the mechanism of the ultimate transfer of iron from the transport globulin to tissue cells has also remained perplexing.

Laurell[3] discussed two major concepts of the transfer of iron to cells. The first view, based on the data of Flexner and associates,[4, 5] assumed that the capillaries are permeable to transferrin, and that the complete iron-transferrin complex leaves the circulating plasma and enters tissue cells. This view was

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considered untenable. The second concept, based on the equilibrium between ionized iron, iron-free transferrin, and the iron-transferrin complex in the plasma as described by Granick,[6] indicated that the ultimate passage of iron through the capillary wall into cells occurred by means of its ionized form. The latter view was preferred by Laurell.

In the present report we wish to consider the state of the serum iron and transferrin after a substantial oral dose of iron given to normal subjects in the fasting state, subjects with hemochromatosis, and to two series of patients with iron deficiency and hypochromic anemia. From the data it will be suggested that transferrin may occur in two phases and that variations in these two phases may explain certain relations between SeFe and TIBC observed in pathologic states. Two abstracts of this material have been previously published.[7, 8]

MATERIALS AND METHODS

Subjects Used in the Study.—The 10 normal subjects were young physicians and technologists in our hospital, 8 were men, 2 were women; all were in good health and aged 20 to 30 years. The 3 patients with iron excess consisted of 2 with idiopathic or endogenous hemochromatosis, and one with exogenous hemochromatosis (iron overload) caused by a chronic hemolytic anemia and more than 200 blood transfusions. Pigmentary cirrhosis or hemosiderosis was demonstrated by liver biopsy in each case. Two series of iron-deficient patients were used. The first group consisted of 17 patients with a background of blood loss, a hypochromic anemia, and hypoferremia.[7] With this first group the serum iron was measured by means of the Barkan and Walker procedure and the UIBC was measured by means of the Schade and Caroline procedure[9] as modified by Rath and Finch.[2]

In presenting the data from this first group,[7] concern was expressed by Moore[10] over the accuracy of the Barkan and Walker method with rising SeFe values. Thus, a second group of 15 patients with a background of blood loss, hypochromic anemia, and hypoferremia were studied.[8] With this second group each serum sample was analyzed simultaneously for iron by 3 methods: the Barkan and Walker method,[11] the method used by Moore and associates,[12] and the method of Kitzes and co-workers.[13] In all instances the UIBC was measured by the Rath and Finch procedure,[2] but in 4 experiments the Feinstein radioactive method[14] was used simultaneously with the Rath and Finch procedure.[2] By analyzing these comparative data statistically it was possible to appraise the Barkan and Walker method in light of other methods, and the Rath and Finch UIBC procedure in light of the Feinstein procedure. Thus, a means of evaluating data obtained by the Barkan and Walker method as used in this laboratory in the first series of patients was evolved.

Hematologic and Other Features (Table I).—One patient (first series) was in the pediatric age group—male, aged 2 years. The remaining patients had ages varying between 19 and 88 years (average 43). Almost two thirds of the patients were women. Of the 32 patients, 25 had severe anemia (hemoglobin, 8 Gm. per 100 ml. or less), 21 revealed a microcytic state (mean corpuscular volume 80 μ^3 or less), and all had hypochromia (mean corpuscular hemoglobin concentration 27 per cent or less in 27). Two patients with macrocytosis (mean corpuscular volume 107 μ^3) had reticulocytosis and polychromatophilia. Six subjects of the first series had SeFe above 50 μg per 100 ml. and seven subjects had TIBC of 300 μg per 100 ml. or less. The other subjects of the first series and all subjects of the second series (total 26) had SeFe less than 50 μg per 100 ml. and TIBC above 350 μg per 100 ml. (total: 26 and 25 subjects, respectively). Bone marrow smears were obtained in one half of the patients and these showed erythroid hyperplasia (normoblastic) and little or no hemosiderin deposits.

The white blood cell and differential counts were normal in all patients. The platelet counts were normal in all but one patient who had idiopathic thrombocytopenia and

metrorrhagia. All patients were afebrile. The blood urea nitrogen was measured in 11 patients (range, 8 to 26 mg. per 100 ml., average 16 mg. per 100 ml.).

The source of blood loss varied, but in over one half of the patients it was distinctly attributable to disturbances in the gastrointestinal tract, menorrhagia, or multiple closely spaced pregnancies.

Plan of Experiment.—Preliminary studies indicated a hypochromic anemia, usually a low SeFe and elevated TIBC, and low per cent saturation of the circulating transferrin. One to several days later the subject received no food for 12 hours. Early the next morning a control sample was obtained and the patient received 1.0 Gm. (first series), or 1.3 Gm. (second series) of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) as granulated crystals in a gelatine capsule plus a glass of water. Nausea and retching were common. Vomiting, however, was usually mild and occurred one to two hours after the oral dose. With the exception of one patient who promptly vomited the entire dose, all patients studied are included in the present report. Thus, no patient selection was made except for the demonstration of the iron-deficient state. The normal subjects and those with hemochromatosis were handled in the same manner. At 1, 2, 4, 6, and 8 hours for the first series, and at 1, 2, and 4 hours for the second series, serum samples were obtained and analyzed. The patient remained in the fasting state throughout in order to keep each serum sample nonlipemic and clear.

Methods.—The hemoglobin was determined by the alkaline and acid hematin methods.[15, 16] The hematocrit reading was determined by Wintrobe's method[17] plus the Chaplin and Mollison[18] correction for trapped plasma. The red cell count was determined by counting 10 squares. The platelet count was obtained by the direct method.

All equipment (syringes, needles, test tubes) was scrupulously acid-washed and rinsed with iron-free water to remove iron contamination.

With the Barkan and Walker procedure the serum was separated from the clot as soon as retraction was definite, and the fresh serum was acidified and kept overnight before proceeding with the development of the color. According to observations of others[20, 21] this procedure with this method yields results comparable to those of other SeFe methods.

The digestion method used by Moore (digesting whole serum) and the method of Kitzes was conducted precisely as given in these references. Since the Moore method uses protein digestion, the serum hemoglobin concentration was determined in 6 experiments. The values in the 31 samples tested varied between 0.08 and 6.4 mg. per 100 ml. according to the benzidine method as used by Crosby and Furth.[22] The mean values in the 6 experiments were 3.2, 2.8, 1.7, 2.0, 2.9, and 4.7 mg. per 100 ml. The amount of iron in these concentrations of hemoglobin would not appreciably alter the results. All samples were grossly clear of hemolysis.

In the Rath and Finch method for UIBC the increment of iron used between readings was 50 μg per 100 ml. The "breaking point" was precise. For the Feinstein method Fe^{59} was used in the form of $FeCl_3$ with a specific activity of about 3.0 mcg. per milligram iron. Six micrograms of iron and 0.02 mcg. of radioactivity were added to 1.0 ml. of serum.

Statistical Analysis.—In all experiments the differences between the zero control and the 2 hour values of the SeFe and TIBC were analyzed by means of a "t" test. It was also determined whether the differences between the values as derived by the Barkan and Walker and Moore values differed significantly from zero ("t" test).

Although all values without selection have been subjected to the statistical analysis, for purposes of presentation and preparation of the figures a gross separation has been made in the data according to one group showing an obvious change in TIBC, and a group showing an equivocal or no obvious change in the TIBC values.

RESULTS

Comparison of Methods.—The Barkan-Walker and Moore methods for serum iron as applied to the serum samples were compared at low (50 μg per 100 ml.), normal (50 to 150 μg per 100 ml.), and elevated (150 to 250, 250 to

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TABLE I. SUMMARY OF HEMATOLOGIC DATA OF TWO SERIES OF IRON-DEFICIENT PATIENTS

SERIES	NO. OF PATIENTS	HGB. (GM./100 ML.)		MCV (μ^3)		MCHC (%)		SFE (μg/100 ML.)		TRANSFERRIN (% SATURATION)	
		RANGE	MEAN ± S.D.	RANGE	MEAN ± S.D.	RANGE	MEAN ± S.D.	RANGE	MEAN ± S.D.	RANGE	MEAN ± S.D.
I	17	2.4–10.4	6.8 ± 1.9	57–107	79 ± 14	19–30	25 ± 1.8	25–90	53 ± 8	6–28	14 ± 6.5
II	15	2.8– 7.7	6.8 ± 2.0	58–107	77 ± 8	19–27	25 ± 2.8	15–50	29 ± 10	3–12	5 ± 2.5

The range, mean, and one standard deviation for each measurement are depicted. MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration.

350+ μg per 100 ml.) serum iron values by analysis of variance. Using all 94 point values, a significant difference was noted between the two methods ($p = 0.005$). This difference was due to the marked difference in the serum samples from 2 subjects (range for these: −54 to −107; range for all other samples: +27 to −19). When these markedly divergent values were deleted by the use of Chauvenet's principle[24] no difference was found between the two methods ($p = 0.4$). No difference was found between the Rath-Finch and Feinstein methods for UIBC ($p = 0.1$) when 24 samples were analyzed. The standard error of the Rath-Finch method on duplicate determinations was 5.9 μg per 100 ml. Comparison of the Barkan-Walker and Kitzes methods and the Moore and Kitzes methods also revealed no significant difference ($p = 0.4$).

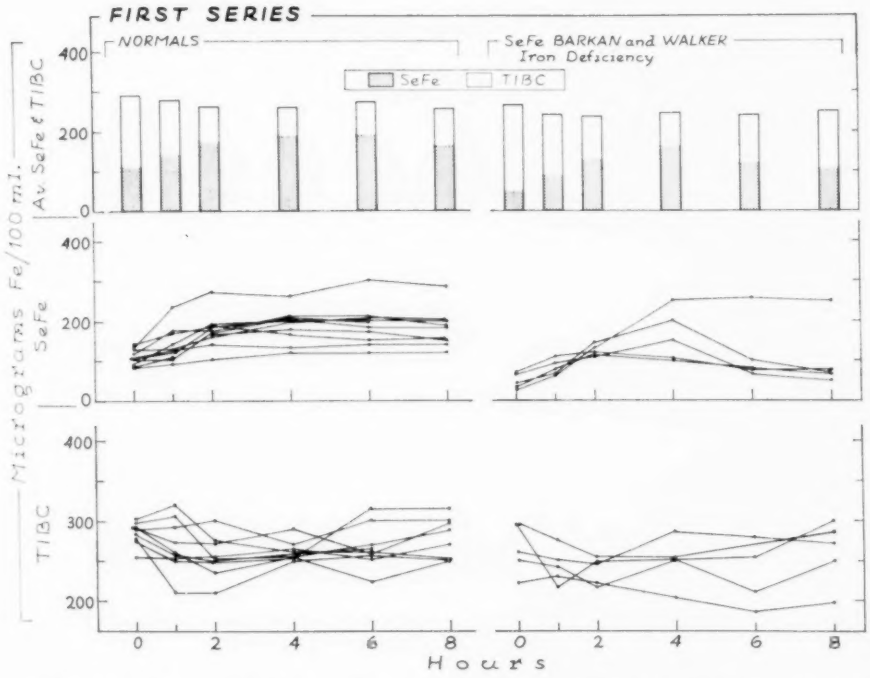

Fig. 1.—The changes in the serum iron and TIBC as obtained by the Barkan and Walker and Rath and Finch methods are shown graphically for the normal subjects and 5 iron-deficient subjects of the first series which depicted minimal changes.

The analyses indicated that the results by the Barkan-Walker method for SeFe as used in this laboratory were as valid as with the Moore and Kitzes methods. Moreover, the Rath-Finch method for UIBC was as satisfactory as the Feinstein method.

Gross Description of Results.—Figs. 1, 2, 3, and 4 depict the results without recourse to statistical analysis.

Figs. 1 and 2 relate all results in the first series in which only the Barkan-Walker method for SeFe was utilized with immediate and overnight acidification

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of the serum plus the Rath-Finch method for UIBC. In this series 1 Gm. of $FeSO_4 \cdot 7H_2O$ was given by mouth after the zero control value and the serum was analyzed for SeFe and UIBC for 8 hours.

Five of the 17 iron-deficient patients (Fig. 1) revealed minimal change in the TIBC at 2 to 4 hours and thereafter (6 to 8 hours). At 2 to 4 hours the decrement varied between –16 and –45 μg per 100 ml. (average –34). These results were similar to those obtained with 10 normal subjects in whom minimal variations were detected (0 to –79, average, –38 μg per 100 ml.). The elevation in SeFe in these groups was similar: for the 5 iron-deficient subjects, the SeFe at 2 to 4 hours varied from the control by +80 to +175 μg per 100 ml. (average, +108), whereas the corresponding values for the 10 normal subjects were +35 to +126 (average, +82).

Viewing the state of the TIBC, after the oral intake of 1 Gm. of $FeSO_4 \cdot 7H_2O$ with the patient in the fasting state, from the standpoint of the 5 iron-deficient subjects selected from a random population of 17, and of the 10 normal subjects, one would be tempted to conclude that the absorption of iron by the gastrointestinal tract does not appear to alter appreciably the TIBC of the circulating plasma. Despite this possibility in these subjects, one could also consider suggestive changes in the TIBC in this group. While the SeFe was elevated, the TIBC in 9 of these 15 subjects was lowered by more than 25

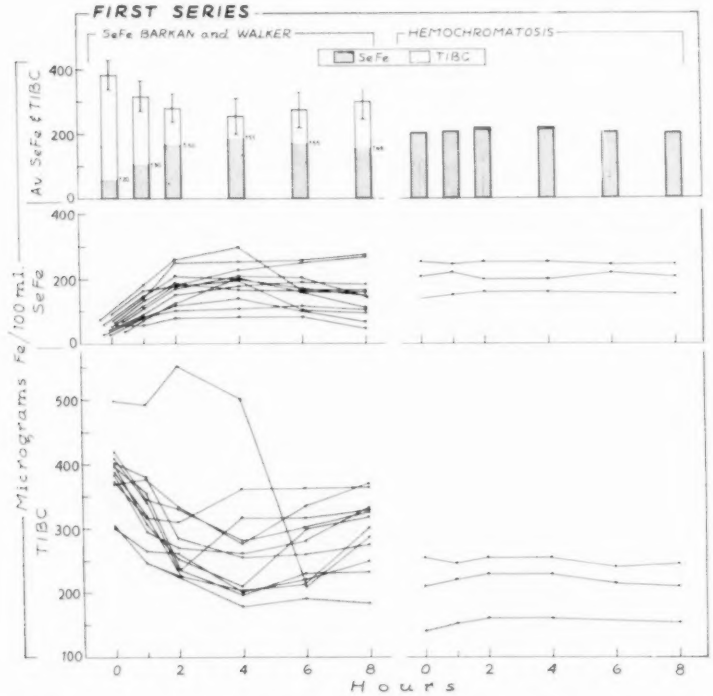

Fig. 2.—The changes in serum iron and TIBC as derived in a manner similar to that of Fig. 1 are shown for 12 iron-deficient patients of the first series who showed a major change in TIBC and for the patients with hemochromatosis.

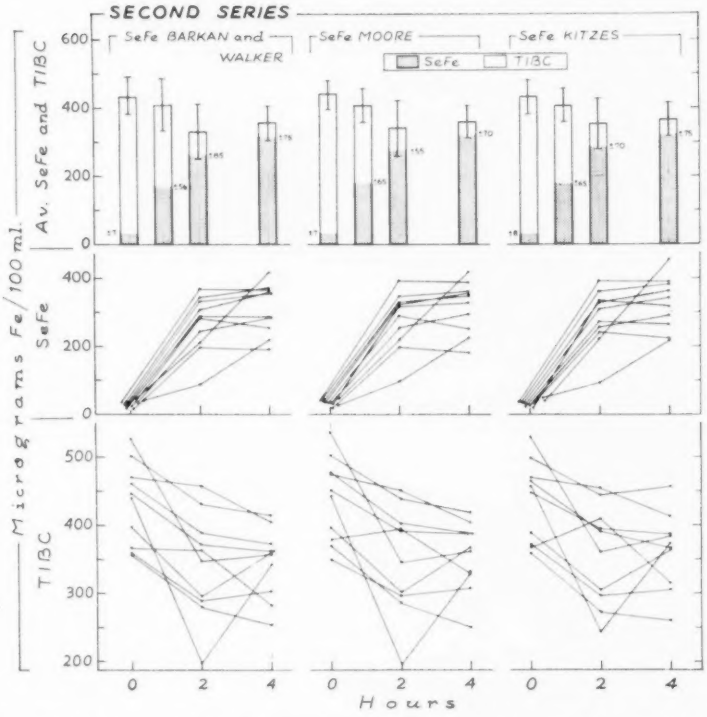

Fig. 3.—This figure contains the changes in serum iron and TIBC for the 10 iron-deficient patients of the second series, and reveal major changes as demonstrated by use of the three serum iron methods and the Rath and Finch UIBC method.

μg per 100 ml. (–30 to –79 μg per 100 ml.) within 2 to 4 hours after 1 Gm. of $FeSO_4 \cdot 7H_2O$. Since the maximal intrinsic error (because of the nature of the technique per se) with the Rath-Finch UIBC procedure tends to be between 25 and 50 μg per 100 ml., some of these figures may indicate a lowering of TIBC if one accepts accuracy for the Barkan-Walker SeFe method as used here.

In Fig. 2 it can be noted that 12 of the 17 iron-deficient subjects of the first series revealed a distinct lowering of the TIBC after they received 1 Gm. of $FeSO_4 \cdot 7H_2O$ orally in the fasting state. In 11 of these patients a definite decrement occurred at 2 hours and in 9 patients this decrement became more pronounced by 4 hours. The only subject in the pediatric age group (aged 2 years) revealed an outstanding lowering of the TIBC at 6 and 8 hours. The average drop in TIBC for these 12 patients was –153 μg per 100 ml. (range –63 to –289 μg per 100 ml.). While this change was in progress the SeFe was elevated by +25 to +226 μg per 100 ml. (average, +137).

In the patients with hemochromatosis there was no change in the SeFe or TIBC after administration of 1 Gm. of $FeSO_4$ by mouth in the fasting state (Fig. 2). The SeFe and TIBC were 210 and 210, 140 and 140, and 254 and 254 in the 3 subjects, and remained near these values for the 8 hours of observation. A small amount of UIBC appeared during the study of the subject with exogenous hemochromatosis.

The second series of 15 iron-deficient patients also could be divided into 2 gross groups based on: (1) an obvious lowering of the TIBC in 2 to 4 hours and (2) minimal or no changes in the TIBC during this interval.

The first subgroup (10 patients) revealed a distinct lowering of the TIBC. This lowering was definite at 2 hours and became more pronounced by 4 hours in 6 cases. Similar results were obtained with the 3 SeFe methods used (Barkan-Walker, Moore, and Kitzes) plus the Rath-Finch UIBC procedure (Fig. 3).

Utilizing the difference in TIBC at 2 to 4 hours from the control (zero) value, the range and mean differences for the 3 methods in these 10 patients were: Barkan-Walker, –64 to –242 (average, –107); Moore, –53 to –246 (average, –107); Kitzes, –55 to –214 (average, –91). The equivalent SeFe values were: Barkan-Walker, +47 to +367 (average, +266); Moore, +67 to +371 (average, +266); Kitzes, +46 to +413 (average +279). All of the values are depicted graphically in the lower panels of Fig. 3.

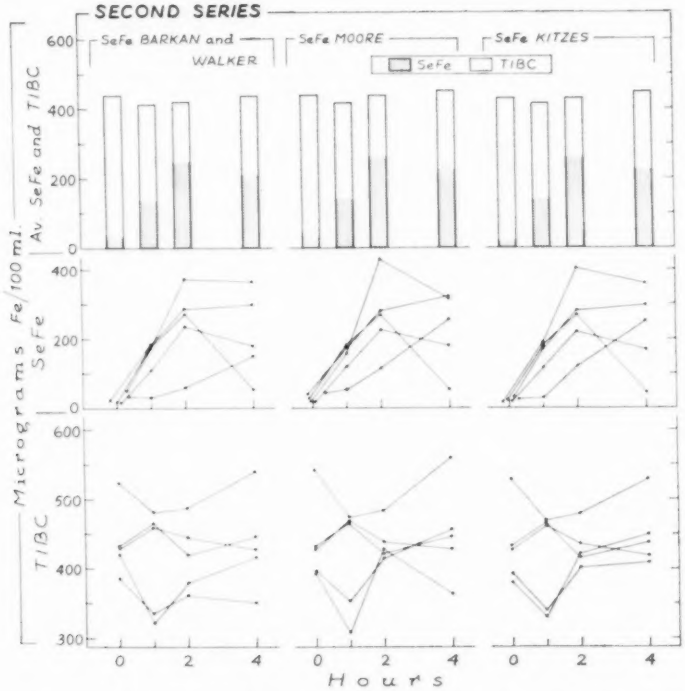

Fig. 4.—Data from the 5 patients of the second series who showed minimal changes by the same approach as that used for the group in Fig. 3 are shown.

Utilizing the mean values for each time interval, thus lumping the results for the patients together, the differences for TIBC were: Barkan-Walker, –105 at 2 hours, and –80 at 4 hours; Moore, –100 at 2 hours, and –80 at 4 hours; and Kitzes, –80 and –70 at 2 and 4 hours, respectively. These data are depicted in the uppermost panel of Fig. 3.

Data from the 5 patients in the second series which revealed minimal or no changes in TIBC are depicted in Fig. 4. The average changes at 2 to 4 hours in this group were: by Barkan-Walker –25, by Moore –16, and by Kitzes +10. Three of these patients showed a decrement in TIBC at one hour amounting to: –100, –50, and –42 by the Barkan-Walker method; –87, –40, and –66 by the Moore method; and –48, –49, and –58 by the Kitzes method. While these observations were made the serum iron was correspondingly elevated over the control (zero) value by +115 to +320 (Barkan-Walker), +79 to 291 (Moore), and +96 to 387 (Kitzes).

The mean values for each time interval for these 5 patients revealed little change (about –25 for TIBC at 1 hour) as shown in the top panel of Fig. 4.

Summary of Gross Separation of Data.—Of the 32 iron-deficient patients in both series, 22 revealed an overt lowering of the TIBC after they had received 1 to 1.3 Gm. of $FeSO_4 \cdot 7H_2O$ by mouth when they were in the fasting state as measured by the Barkan-Walker SeFe method and the Rath-Finch method for UIBC. Ten of these patients had the results confirmed by other methods. The remaining patients (10) revealed an equivocal lowering or no change of TIBC. Results similar to those of the latter 10 patients were obtained with 10 normal subjects. In hemochromatosis no changes in TIBC were noted.

Simultaneous Measurement of UIBC; Rath-Finch and Feinstein Methods.—Table II contains data from 4 iron-deficient subjects of the second series who received 1.3 Gm. $FeSO_4 \cdot 7H_2O$ by mouth when they were in the fasting state and in whom SeFe was measured by the 3 methods and UIBC by the 2 methods. It is to be noted that the results were quite similar irrespective of the methods used. In 2 to 4 hours the TIBC was lowered in 3 patients (–87 to –111, –137 to –180, and –177 to –220) and not altered in one (–20 to +12).

Complete Saturation of Transferrin During Iron Absorption.—The second and third cases of Table II demonstrated complete saturation of the circulating transferrin 2 to 4 hours after the oral dose of iron (1.3 Gm. $FeSO_4$). Under these conditions the indirect measurement of the transferrin concentration in the 2 to 4 hour sample became essentially dependent on the measurement of the serum iron concentration, since with this situation there was no change in the spectrophotometric reading with the addition of ionized iron in the Rath-Finch procedure for UIBC and no change in residual radioactivity in the Feinstein procedure for UIBC. Conversely, the control (zero hour) value was dependent mainly on the UIBC procedure since the control SeFe was low (per cent saturation of transferrin 5 to 9 per cent). In these cases the TIBC was prominently lowered. It may be inferred that this type of measurement of altered TIBC has additional significance since it is dependent primarily on one instead of two types of indirect analysis (a high UIBC and very low SeFe for the control, and a high SeFe and no UIBC for the test value).

For the entire series of 32 iron-deficient patients tested, 12 developed complete saturation of the circulating transferrin 2 to 4 hours after the large oral

TABLE II. DATA RELATING MEASUREMENT OF SERUM IRON AND TIBC ON SAME SAMPLE BY MULTIPLE METHODS

PATIENT	SAMPLE (HR.)	SeFe (μg/100 ML.)			TIBC (μg/100 ML.)					
					B-W		M		K	
		B-W	M	K	R-F	F	R-F	F	R-F	F
1	0	24	33	27	461	455	470	463	464	457
	0.33	74	68	71	464	443	452	437	461	440
	0.66	133	141	135	463	470	471	478	461	468
	1.0	187	202	192	463	449	477	464	467	454
	2.0	307	321	311	387	374	401	388	391	378
	4.0	349	359	341	374	354	384	364	366	346
2	0	27	36	29	527	516	536	525	529	518
	0.33	66	71	63	506	508	511	513	503	505
	0.66	176	175	169	476	467	475	466	469	460
	1.0	256	238	258	456	439	438	421	458	441
	2.0	347	345	360	347	359	345	357	360	378
	4.0	364	362	381	364	364	362	362	381	381
3	0	24	33	23	359	351	368	360	358	350
	0.33	27	41	29	352	341	366	355	354	343
	0.66	74	79	79	349	341	354	346	351	343
	1.0	140	141	138	325	310	326	311	312	308
	2.0	279	283	272	279	296	283	300	272	289
	4.0	253	250	260	253	273	250	270	260	280
4	0	22	26	23	432	442	436	446	433	443
	0.33	32	30	31	445	454	443	452	444	453
	0.66	108	110	112	458	453	460	455	462	457
	1.0	174	178	179	464	453	468	457	469	458
	2.0	271	273	269	421	439	423	441	419	437
	4.0	54	56	49	444	428	446	430	439	423

The serum iron method included the Barkan-Walker (B-W), the Moore (M), and the Kitzes (K) methods. TIBC was measured by the Rath and Finch (R-F) and the Feinstein (F) plus each of the three serum iron methods.

TABLE III. STATISTICAL ANALYSIS OF DATA USING THE BARKAN AND WALKER SERUM IRON METHOD

VALUE	TIBC (μg/100 ML. MEAN VALUE)		
	NORMAL	Fe DEFICIENCY	P
Control	289	390	0.001
4 hr.	261	316	0.005
Control to 4 hr.	−28	−74	0.001

For purposes of this discussion the change at 4 hours was chosen.

dose of iron (5 in the first series and 7 in the second series). This group of 12 patients revealed a lowering of TIBC which averaged −116 μg per 100 ml. (−40 to −242 μg per 100 ml.). The control sample for this group had a SeFe which averaged 35 μg per 100 ml. (15 to 70), and a per cent saturation of transferrin which averaged 8.5 per cent (3 to 20 per cent). It should be noted that in the 7 patients of the second series the SeFe was dependent on 3 methods, and for 2 of these patients the UIBC was dependent on 2 methods.

Statistical Analysis.—Table III contains an analysis of the changes in TIBC as detected by the Barkan and Walker SeFe method and the Rath and Finch UIBC method. Other methods yielded similar results. In this analysis all values obtained were used without selection. There was a significant difference in the TIBC between the normal and the iron-deficient groups before (control value) and 4 hours after the oral dose of iron. The lowering of TIBC in the

iron-deficient subjects exceeded the intrinsic errors of the procedures used, and was significantly different from the control (zero) value. Although the lowering of TIBC 4 hours after the oral dose in the normal subjects also differed significantly from the control value, the values derived were frequently within the error of the procedures used.

DISCUSSION

The results herein recorded indicate a lowering of the transferrin concentration of the circulating plasma in patients in the iron-deficient state under the impact of a large oral dose of iron given during a period of fasting. Thus, it appears that transferrin need not remain in a relatively fixed concentration in the circulating plasma. Rather, the results suggest mechanisms for sudden changes in the concentration of circulating transferrin under special, and possibly limited, circumstances. The occurrence of substantial and rapid changes in the circulating transferrin concentration under special circumstances suggests the possibility of rapid yet limited changes transpiring in the homeostatic physiologic state.

An explanatory hypothesis may be offered for the significant results derived by the present experiments. Such a hypothesis should attempt to account for the following findings: (1) the low SeFe and high TIBC encountered in patients deficient in iron and the high SeFe and lowered TIBC observed in patients with hemochromatosis; (2) the decrement in TIBC while the SeFe is elevated during a major period of iron absorption in iron-deficient patients, which effects a shift in the SeFe-TIBC relationship from a low SeFe–high TIBC to a high SeFe–lowered TIBC, or a change from the configuration of iron deficiency to that of hemochromatosis; (3) no change in the SeFe-TIBC relationship after a large oral dose of iron in patients with hemochromatosis; and (4) minimal or no change in TIBC during iron absorption in the normal state.

The hypothesis herein offered considers transferrin to exist in two phases, one circulating generally in the plasma and the other sequestered at cellular sites. An equilibrium between these two phases of transferrin may be visualized which, in the normal steady state, is reflected by the normal range of SeFe and TIBC. Under circumstances wherein the body iron and/or iron metabolism is altered to a significant degree, shifts in the relationship between circulating and sequestered transferrin may be considered to occur. Thus, it is conceivable that in iron deficiency the sequestered portion of transferrin is lowered while the circulating portion increases because primarily of a diminution of iron turnover at cellular sites. A major burst of iron absorption in the iron-deficient state may shift the circulating transferrin temporarily to cellular sites as iron moves from plasma to cells and the sequestered component increases at the expense of the circulating portion. Such a concept would explain the rapid change in the iron transport system from the iron-deficient to the hemochromatotic configuration as observed in the present experiments.

The hypothesis which postulates a balance between circulating and sequestered phases of transferrin is at odds with the proposals made by Laurell and

by Flexner and co-workers concerning the means by which iron is ultimately transferred from plasma into cells. Flexner and co-workers interpreted their data as indicating a consumption of transferrin by cells in the catabolic sense while iron was released within the cells. Laurell argued against the transfer of such a large complex across membranes, capillary and cellular, as well as the high rate of turnover of the complex globulin transferrin demanded by Flexner's view. Laurell's hypothesis considered the ultimate transfer of iron across membranes into cells as ions under the regulation of mass action principles. Laurell's position may be considered as primarily based on two features: (1) Granick's treatment of the dissociation of the iron-transferrin complex and (2) Laurell's data which indicate no change in the plasma transferrin concentration in various conditions associated with a rapid elevation of SeFe, including the absorption of iron given orally to normal and iron-deficient subjects.

Granick's data which concern the ionization of the iron-transferrin complex need not pertain directly to iron transportation into cells in all biologic systems. The lack of observance of a lowering of transferrin during iron absorption in iron deficiency by Laurell is more disconcerting with respect to the circulating-sequestered transferrin hypothesis. It is noted, however, that a significant lowering of the transferrin concentration has been difficult to demonstrate in the normal state and in one third of iron-deficient subjects in the presently reported observations when the oral iron dose was greater than that used by Laurell by a factor of 3 to 4. Among the latter one third group there were patients who displayed a lowered transferrin value which was not significant by the methods available and used, and there were patients who revealed a significant lowering of transferrin concentration but only during the early period of iron absorption. It is possible that certain differences in the data related by Laurell and the data herein presented may have been due to the timing of the observations, the dose of iron used, and the selection of patients.

The lack of change in the transferrin concentration when oral iron was given to patients with hemochromatosis who were in the fasting state requires an explanation consistent with the circulating-sequestered transferrin hypothesis. In advanced iron overload the rate of iron absorption may be diminished as indicated by Bothwell and associates,[25] and such a circumstance would alter the results of the present study. Assuming that iron absorption occurred in our patients as it usually does in patients with hemochromatosis,[25] the lack of change in SeFe-TIBC could have resulted from a complete or near complete saturation of cellular sites by the iron-transferrin complex, or, stated in terms of the hypothesis, a maximum degree of sequestration of transferrin may have been present. Under these conditions, in order for iron to move into cells the hypothesis would suggest that a circulating molecule of transferrin replaces a sequestered molecule while the circulating concentration remains unchanged.

Our data agree with that of Laurell as these relate to the normal state. Either no change occurred in the transferrin concentration or a decrement occurred which was within the possible error of the procedure used. These data,

however, need not negate the concept of a circulating and a sequestered phase of transferrin. During iron absorption the level of SeFe must be dependent on the iron flux, that is the influx from the bowel and the efflux into cells. Thus, the rate of movement of iron during absorption in the normal state may not equal that in iron deficiency, and, in terms of the circulating-sequestered transferrin hypothesis, this could mean a lesser degree of sequestration of transferrin and, in turn, a lesser degree of lowering of its concentration in the circulating plasma.

Assuming that transferrin is partly in a sequestered phase, by what means may such be visualized? The most likely sites of sequestration would be expected to be in tissues which have a high iron turnover such as liver, spleen, and bone marrow. These sites have been considered by anatomists as having an open circulation by means of which plasma may come into direct contact with cells which are entering into iron metabolism. It is proposed that at these sites the ultimate transfer of iron into cells is accomplished by an orientation of the iron containing transferrin molecule to iron receptors on the surface of the cell followed by iron transfer and that such orientation and iron transfer requires time. The time interval for such orientation and transfer would be reflected by a sequestered phase of transferrin.

The hypothesis which considers transferrin to exist in two phases, one circulating and the other at cellular sites, offers a unitarian view concerning the relationship of SeFe to TIBC in iron deficiency and hemochromatosis. In iron deficiency the cell iron, particularly in the liver, spleen, and bone marrow, is reduced. The serum iron concentration (SeFe) is reduced, the movement of iron is retarded, and, according to the hypothesis, the cell receptors for iron are relatively inactive, and are releasing transferrin to the general circulation. The circulating transferrin is thus considered to be elevated, which causes a prominent lowering of its saturation with iron. In hemochromatosis the converse is considered to pertain: cell iron is high, serum iron is high, and the movement of iron is substantial. The hypothesis considers the cell receptors for iron to be very active, thus tending to remove transferrin from the general circulation and further elevating the saturation of circulating transferrin with iron.

It is conceivable that other variations in the SeFe-TIBC relationship observed in abnormal states may be explainable by the circulating-sequestered transferrin hypothesis. In this connection recent data by Colehour and associates[26] may be cited. These authors found that acute anoxia of the rat caused a rapid increment in TIBC. These observations demonstrated by another approach the lack of fixation of the transferrin concentration and the fact that mechanisms are available for rapid changes in its concentration. In accordance with the circulating-sequestered transferrin hypothesis it may be considered that anoxia releases sequestered transferrin into the general circulation.

SUMMARY

1. When 1.0 or 1.3 Gm. of $FeSO_4 \cdot 7H_2O$ was given orally to iron-deficient patients in the fasting state, the total iron binding capacity (TIBC) of the

serum of 22 of the 32 patients was significantly lowered within 2 to 4 hours. Return of the TIBC toward normal was observed after 6 to 8 hours. An additional 4 patients displayed a significant lowering of the TIBC for one hour after the oral dose of iron. The remaining 6 patients with iron deficiency revealed a negligible change or no change in TIBC after the oral dose.

2. When 1.0 Gm. of $FeSO_4 \cdot 7H_2O$ was given orally in the fasting state to normal subjects or patients with hemochromatosis, negligible change or no change occurred in the TIBC.

3. The serum iron concentration (SeFe) was distinctly elevated in all but 2 of 42 normal and iron-deficient subjects given iron orally (1 to 1.3 Gm.) while they were in the fasting state. The SeFe remained unchanged when oral iron was given to patients with hemochromatosis.

4. The lowering of TIBC observed in iron deficient patients after receiving iron orally was the same when 3 methods for SeFe and 2 methods for unsaturated iron binding capacity (UIBC) were used simultaneously. No significant difference was noted in the measurement of SeFe by 3 methods (Barkan and Walker, Moore, and Kitzes methods). No significant difference was noted in the measurement of UIBC by 2 methods (Rath-Finch and Feinstein methods).

5. In 12 of the 32 iron-deficient subjects the lowering of the TIBC during iron absorption was associated with complete saturation of the circulating transferrin with iron.

6. The results presented indicate the existence of mechanisms which effect sudden changes in the circulating transferrin concentration.

7. An explanatory hypothesis for the results obtained has been offered. The hypothesis considers transferrin to exist in two phases, one circulating in the plasma and the other sequestered at certain cellular sites. Furthermore, it is proposed that sequestration may result from orientation of iron laden transferrin to iron receptors at cellular sites during the transferral of iron to cells.

REFERENCES

1. Laurell, C. B.: Plasma Iron and the Transport of Iron in the Organisms, Pharmacol. Rev. **4**: 371, 1952.
2. Rath, C. E., and Finch, C. A.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. XXXVIII. Serum Iron Transport. Measurement of Iron-Binding Capacity of Serum in Man, J. Clin. Invest. **28**: 79, 1949.
3. Laurell, C. B.: What Is Function of Transferrin in Plasma? Blood **6**: 183, 1951.
4. Flexner, L. B., Vosburgh, J., and Cowie, D.: Capillary Permeability: Rate of Transcapillary Exchange of Iron Added to Plasma as Radioactive Ferric Betaglobulinate, Am. J. Physiol. **153**: 503, 1948.
5. Flexner, L. B., Cowie, D. B., and Vosburgh, J.: Studies on Capillary Permeability With Tracer Substances (Cold Spring Harbor Symposia), Quant. Biol. **13**: 88, 1948.
6. Granick, S.: Iron Metabolism, Bull. New York Acad. Med. **30**: 81, 1954.
7. Mitchell, J. H., Halden, E. R., Bryan, S., and Muirhead, E. E.: Response of the Iron-Transport System to the Ingestion of Ferrous Sulfate, J. LAB. & CLIN. MED. **48**: 926, 1956. (Abst.)
8. Muirhead, E. E., Halden, E. R., Mitchell, J. H., Stirman, J. A., and Jones, F.: Sequestration of Transferrin During Iron Absorption in Iron Deficiency, J. LAB. & CLIN. MED. **50**: 935, 1957. (Abst.)

9. Schade, A., and Caroline, L.: An Iron-Binding Component in Human Blood Plasma, Science **104:** 340, 1946.
10. Moore, C. V.: Comments at meeting of Central Society for Clinical Research (Chicago, November, 1956) on presentation summarized in reference number 7 of this paper.
11. Barkan, G., and Walker, B. S.: Determination of Serum Iron and Pseudo-hemoglobin Iron With O-Phenanthroline, J. Biol. Chem. **135:** 37, 1940.
12. Moore, C. V., Arrowsmith, W. R., Quilligan, J. J., and Read, J. T.: Studies in Iron Transportation and Metabolism. I. Chemical Methods and Normal Values for Plasma Iron and "Easily Split Off" Blood Iron, J. Clin. Invest. **16:** 613, 1937.
13. Kitzes, G., Elvehjem, C. A., and Schuette, H. A.: The Determination of Blood Plasma Iron, J. Biol. Chem. **155:** 653, 1944.
14. Feinstein, A. R., Bethard, W. F., and McCarthy, J. D.: A New Method Using Radioiron, for Determining the Iron-Binding Capacity of Human Serum, J. LAB. & CLIN. MED. **42:** 907, 1953.
15. Cohen, B., and Smith, A. H.: The Colorimetric Determination of Hemoglobin, J. Biol. Chem. **39:** 489, 1919.
16. Clegg, J. W., and King, E. J.: Estimation of Haemoglobin by the Alkaline Haematin Method, Brit. M. J. **2:** 329, 1942.
17. Wintrobe, M. M.: A Simple and Accurate Hematocrit, J. LAB. & CLIN. MED. **15:** 287, 1929.
18. Chaplin, H., and Mollison, P. L.: Correction for Plasma Trapped in the Red Cell Column of the Hematocrit, Blood **7:** 1227, 1952.
19. Dameshek, W.: A Method for the Simultaneous Enumeration of Blood Platelets and Reticulocytes, Arch. Int. Med. **50:** 579, 1932.
20. Peters, T., Giovanniello, T. J., Apt, L., and Ross, J. F.: A Simple Improved Method for the Determination of Serum Iron. II, J. LAB. & CLIN. MED. **48:** 280, 1956.
21. Cartwright, G. E., Huguley, C. M., Ashenbrucker, H., Fay, J., and Wintrobe, M. M.: Studies on Free Erythrocytes, Protoporphyrin, Plasma Iron and Plasma Copper in Normal and Anemic Subjects, Blood **3:** 501, 1948.
22. Crosby, W. H., and Furth, F. W.: A Modification of the Benzidine Method for Measurement of Hemoglobin in Plasma and Urine, Blood **11:** 380, 1956.
23. Croxton, F. E.: Elementary Statistics With Applications in Medicine, New York, 1953, Prentice-Hall, Inc.
24. Worthing, A. G., and Geffner, J.: Treatment of Experimental Data, New York, 1943, John Wiley & Sons, Inc., pp. 170-171.
25. Bothwell, T. H., van Doorn-Wittkampf, H., van W. Du Preez, M. L., and Alper, T.: The Absorption of Iron. Radioiron Studies in Idiopathic Hemochromatosis, Malnutritional Cytosiderosis, and Transfusional Hemosiderosis, J. LAB. & CLIN. MED. **41:** 836, 1953.
26. Colehour, J. K., Borsook, H., and Graybrill, H.: Effect of Hypoxia on Serum Iron and the Unsaturated Iron-Binding Capacity of Serum in Rats, Am. J. Physiol. **191:** 113, 1957.

URINARY EXCRETION OF CHLORPROMAZINE IN MAN

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Patients who had been maintained on metabolic wards for 5 years were given chlorpromazine orally in doses of 300 mg. and 800 mg. daily. The ether soluble fraction of chlorpromazine excreted in the urine was determined and was found to be 7 to 9 per cent of the total chlorpromazine administered. Determinations were carried out after medication had been stopped to establish the length of time that this ether soluble fraction was excreted. The latter was found to be excreted in three forms: bound chlorpromazine, free chlorpromazine sulfoxide, and bound chlorpromazine sulfoxide. There was a significant drop in the excretion of chlorpromazine and chlorpromazine sulfoxide the first day after cessation of medication, and a further decrease was observed on the second day. Thereafter, the elimination continued for 4 days in small but measurable amounts and this occurred on a dosage of 800 mg. per day, whether it had been given for 4 or 11 days. The results indicate that the continued salutary behavioral results which may be observed after the first two days following cessation of medication may be due to factors other than a significant amount of chlorpromazine which still remains in the body.

IN this investigation of the metabolism of chlorpromazine two methods for the determination in the urine were used. One is the semiquantitative determination reported by Forrest and collaborators.[1] The other is on a quantitative basis by the procedure of Flanagan and co-workers[2] to determine the ether soluble metabolites of chlorpromazine. Armed with these methods it became possible to study several problems in chlorpromazine metabolism. For example, how rapidly is that drug destroyed in the body? An idea of the rate of destruction could be obtained by the duration of the period in which chlorpromazine and chlorpromazine products could be recovered in the urine. Sometimes behavioral improvement evoked by chlorpromazine disappears soon after the medication is stopped. In other instances improvement continues for a longer or shorter time after cessation of drug administration. What relationship exists between the changes in symptoms and the presence or absence of chlorpromazine in the body? An attempt to answer these and similar questions was the purpose of this study.

METHOD

The details of the semiquantitative method are available.[1] The principle of the test depends upon the development of a violet color which is used to measure chlorpromazine in the urine. The reaction concerns a relatively unstable metabolite with ferric ions in

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an acid medium. Indications are that this metabolite is an intermediate between chlorpromazine and its sulfoxide. The metabolite is a reactive and photosensitive compound and the color derived therefrom is unstable. The violet color used for reading gradually changes into reddish shades because of oxidoreduction processes.

In the quantitative test[2] for chlorpromazine in the urine the free base of chlorpromazine is extracted from an alkaline medium with ether. The extract is washed with alkali and water to remove impurities. The chlorpromazine is extracted from the ether with 1N H_2SO_4 and the ultraviolet absorption caused by the acid salt of free chlorpromazine and its sulfoxide is measured spectrophotometrically over a range of 220 to 320 mμ. The urine residue from the ether extract is subjected to an alkaline hydrolysis and the additional chlorpromazine released is referred to as the "bound" chlorpromazine and its sulfoxide. A determination is made by following the above procedure for free chlorpromazine and its sulfoxide.

Schizophrenic patients who have been maintained for a period of at least 5 years on research wards were the subjects for these experiments. Trained personnel made it possible to collect complete 24 hour urine specimens. Three series of experiments were made: (a) two patients on a dosage of 300 mg. of chlorpromazine daily (75 mg. 4 times daily) for 4 days, (b) two patients on a dosage of 800 mg. chlorpromazine (200 mg. 4 times daily) for 4 days, and (c) same patients as in experiment (b) but medication was continued for a total of 11 days. In each series urine samples were analyzed during medication and after administration of the drug had been stopped. These analyses were continued until chlorpromazine and chlorpromazine sulfoxide failed to appear in the urine. Additional patients who, however, also had not previously been taking this drug were placed on 75 mg. of chlorpromazine 4 times daily. Twenty-four hour urine collections were started the morning the first dose of chlorpromazine was administered. We determined the ether soluble extract of chlorpromazine and found it to be excreted in three forms: bound chlorpromazine, free chlorpromazine sulfoxide, and bound chlorpromazine sulfoxide.

RESULTS

The two patients placed on 300 mg. chlorpromazine daily excreted an average of 3.2 per cent of the total chlorpromazine given in the first 24 hours. Maximum excretion was obtained during the second 24 hour period and remained approximately the same while chlorpromazine medication was continued. One patient excreted between 5 and 6 L. of urine daily. No chlorpromazine was detected the first two days probably because of the great urinary dilution; thereafter 1.8 to 3.6 per cent was present in the urine.

Two other patients who were placed on 200 mg. chlorpromazine 4 times daily for 4 days revealed recoveries of approximately 6.0 per cent in the first 24 hours. Beginning with the second day, recovery was found to be between 7.0 and 9.0 per cent and remained in the same range as long as patients were on medication. Bound chlorpromazine was present in the urine for 48 hours, whereas free and bound sulfoxide were present for 6 days after cessation of medication. There was probably excretion of chlorpromazine sulfoxide after this time but detection of it was not possible by the procedure used. Two months later these same two patients were again placed on 200 mg. chlorpromazine 4 times daily and kept on medication for a total of 11 days. The amount of ether soluble chlorpromazine and its sulfoxide excreted in the urine per day was approximately the same as in the earlier experiment. The excretion of the free unchanged drug was negligible. Fig. 1 shows the recovery of chlorpromazine and chlorpromazine sulfoxide in terms of milligrams. Each

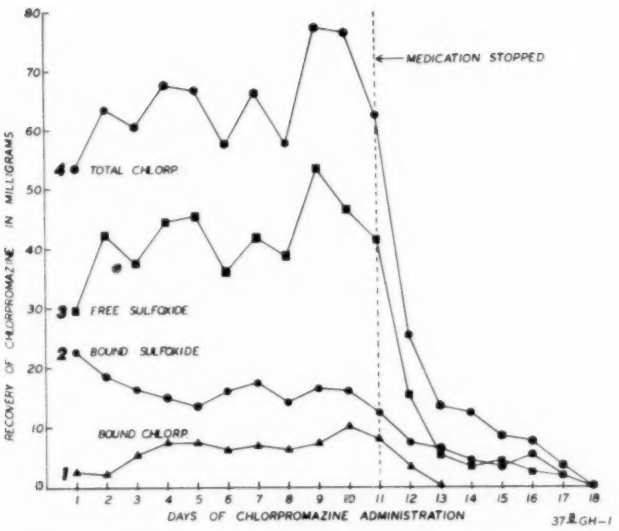

Fig. 1.—Recovery of chlorpromazine and chlorpromazine products from a patient receiving 800 mg. of chlorpromazine daily. Abscissas represent days from start of medication. Ordinates are in milligrams of chlorpromazine. *1*, Excretion of bound chlorpromazine. *2*, Excretion of bound chlorpromazine sulfoxide. *3*, Excretion of free chlorpromazine sulfoxide. *4*, Sum of *1*, *2*, and *3*.

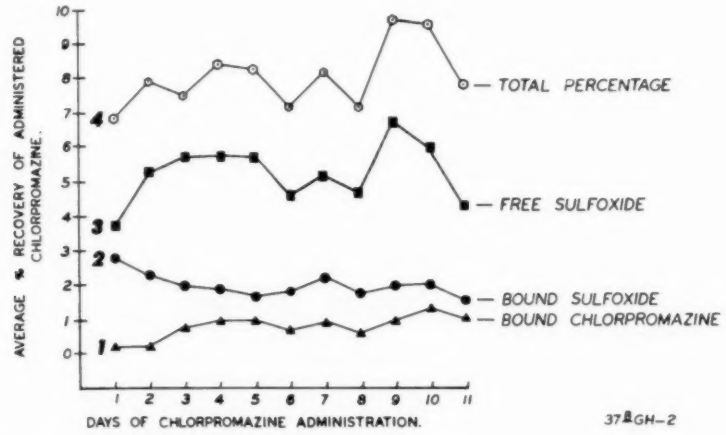

Fig. 2.—Comparison of average recovery percentages of administered chlorpromazine as chlorpromazine and chlorpromazine sulfoxide. Abscissas represent days from start of medication. Ordinates are in percentage recovery of administered chlorpromazine. *1*, Per cent of bound chlorpromazine. *2*, Per cent of bound chlorpromazine sulfoxide. *3*, Per cent of free chlorpromazine sulfoxide. *4*, Sum of *1*, *2*, and *3*. Free chlorpromazine sulfoxide is excreted in higher concentrations than the other two urinary componets of chlorpromazine.

point represents the actual amount of ether soluble chlorpromazine and chlorpromazine sulfoxide excreted in a 24 hour period. The total chlorpromazine curve is the sum of the findings for bound chlorpromazine, free sulfoxide, and bound sulfoxide. The major component recovered was chlorpromazine sulfoxide in the free and bound states. After cessation of medication, chlorpromazine in the bound form disappeared from the urine at the end of two days, whereas free and bound sulfoxide continued to be excreted for an additional 4 days.

The presence of chlorpromazine sulfoxide in the urine for 6 days after medication had been stopped may be due to some accumulation of the drug in the body.

Fig. 2 represents the average percentages of ether soluble chlorpromazine and chlorpromazine sulfoxide excreted in the urine during administration of the drug. The total curve represents the sum of the percentage of bound chlorpromazine, free chlorpromazine sulfoxide, and bound chlorpromazine sulfoxide.

The Forrest[1] test was performed on each 24 hour specimen of urine and the maximum reading was obtained on the third day during the administration of the drug. The test became negative the third day after medication had been stopped.

DISCUSSION

The experiments described indicate that ether soluble chlorpromazine and chlorpromazine sulfoxide are excreted in the urine of man in amounts of from 7.0 to 9.0 per cent of an administered dose of 200 mg. given 4 times daily. Patients taking 75 mg. of chlorpromazine 4 times daily excreted ether soluble chlorpromazine and chlorpromazine sulfoxide in the urine in amounts from 5.5 to 7.0 per cent of the administered dose. There is probably no significant difference with the excretion in the urine at these two dosage levels. The Forrest test[1] remains positive for 3 days, after which it becomes negative, whereas quantitative assay still shows chlorpromazine metabolites. This discrepancy suggests that the Forrest[1] test may possibly be based on a chlorpromazine metabolite other than chlorpromazine sulfoxide. In any event, according to Salzman and Brodie,[3] the sulfoxide of chlorpromazine is widely metabolized in the various organs of the body. Two patients receiving 900 mg. chlorpromazine orally per day excreted negligible amounts of unchanged drug with about 5 per cent eliminated as the sulfoxide.[3] Van Loon[4] recovered about 50 per cent of the administered dose (10 mg. per kilogram) of chlorpromazine S^{35} in the feces of rats with greatest concentration in the second 24 hour period when administered by stomach tube. The drug was administered at a dose of 10 mg. per kilogram of chlorpromazine S^{35} and the total recovery of S^{35} in the urine and feces for 4 days after the administration of the radioactive material was approximately 96 per cent. Apparently in rats the urinary excretion of chlorpromazine occurs more rapidly and in larger amounts than in human subjects. Nadeau and Sobolewski[5] hydrolyzed the chlorpromazine conjugate with β-glucuronidase and found this method to be more accurate than chemical hydrolysis.

In the metabolism of chlorpromazine the product formed from the sulfoxide may be the sulfone, but there may even be an earlier intermediary metabolite than the sulfoxide. Another suggested method for the metabolites consists of the demethylation of chlorpromazine. Ross[6] found the rat capable of demethylating chlorpromazine C^{14} after oral or intravenous administration of the drug. Salzman and Brodie,[3] however, failed to detect any demethylation

of chlorpromazine upon incubation of the drug with rabbit-liver homogenate, yet La Du[7] showed an active demethylating system for drugs to be present in the liver.

Our observation discloses that the excretion of chlorpromazine has decreased significantly during the first 48 hours after cessation of medication, which indicates that any behavioral improvement in patients cannot be attributed to the presence of a significant amount of chlorpromazine in the body after the second day following withdrawal of the drug. Rinaldi and associates[8] studied a group of 39 patients, 32 of whom were schizophrenic, 6 schizoaffective, and one with involutional melancholia, all with long histories of hospitalization. In most patients signs of improvement decreased noticeably 2 days after medication had been stopped, but in some patients beneficial effects were sustained for longer periods. The brief duration of the salutary therapeutic results after cessation of medication can be explained in part by the rapid elimination of chlorpromazine by the body. At two different dose levels the recovery of the ether soluble fraction of chlorpromazine in the urine was approximately the same. This suggests that the ether soluble portion is a relatively constant fraction of the total chlorpromazine in the body. If this should prove to be the case then the ether soluble fraction should be an indicator of the total chlorpromazine thus suggesting that not only the ether soluble fraction but most of the chlorpromazine is excreted from the body within the first few days after ingestion. Van Loon[4] recovered 96 per cent of administered chlorpromazine S^{35} in the feces and urine of rats within 96 hours after administration. But this rapid excretion may be explained by a species difference, for, in general, small animals have faster metabolic rates.

SUMMARY

Three major urinary components of ether soluble chlorpromazine: chlorpromazine sulfoxide in the free and bound forms and bound chlorpromazine were eliminated in 5.5 to 7.0 per cent amounts when patients were receiving 200 mg. daily. On a higher dosage of 800 mg. daily, the urinary excretion amounts to 7.0 to 9.0 per cent of the ingested chlorpromazine. There was a significant drop in the excretion of chlorpromazine and chlorpromazine sulfoxide the first day after cessation of medication, and a further decrease was observed on the second day. Thereafter, the elimination continued for 4 days in small but measurable amounts and this occurred on a dosage of 800 mg. per day, whether it had been given for 4 or 11 days. It may, therefore, be concluded that chlorpromazine is rapidly metabolized and eliminated by the body. These results indicate that the continued salutary behavioral results which may be observed after the first two days following cessation of medication may be due to factors other than a significant amount of chlorpromazine still remaining in the body.

I am indebted to Dr. Harold E. Himwich for constant advice and criticism during this study.

REFERENCES

1. Forrest, F. M., Forrest, Irene M., and Mason, A. S.: A Rapid Urinary Test for Chlorpromazine, Promazine and Pacatal, Am. J. Psychiat. **113**: 931, 1957.
2. Flanagan, T. L., Lin, T. H., Novick, W. J., Rondish, I. M., Bocher, C. A., and Van Loon, E. J.: Spectrophotometric Method for the Determination of Chlorpromazine and Chlorpromazine Sulfoxide in Biological Fluids, J. M. & Pharm. Chem. **1**: 3, 1959.
3. Salzman, N. P., and Brodie, B. B.: Physiological Disposition and Fate of Chlorpromazine and a Method for Its Estimation in Biological Material, J. Pharmacol. & Exper. Therap. **118**: 46, 1956.
4. Van Loon, E. J.: Personal communication of preliminary experiments.
5. Nadeau, G. D., and Sobolewski, G.: Canad. M. A. J. **80**: 1959.
6. Ross, J. J., Jr., Young, R. L., and Maass, A. F.: Demethylation of Chlorpromazine (n-Methyl)-C^{14}, Science **128**: 1279, 1958.
7. La Du, B. N., Gaudette, L., Trousol, N., and Brodie, B. B.: Enzymatic Dealkylation of Aminolyrine (Pyramidon) and Other Alkylamines, J. Biol. Chem. **214**: 741, 1955.
8. Rinaldi, R., Rudy, L. H., and Himwich, H. E.: Clinical Evaluation of Azacyclonol, Chlorpromazine and Reserpine on a Group of Chronic Psychotic Patients, Am. J. Psychiat. **112**: 678, 1956.

THE PRESERVATION OF BLOOD FOR TRANSFUSION

IV. In Vitro Recovery After Freezing and Thawing of Red Cells Modified With Sugars

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This report deals with the effect of dextrose and lactose, alone or in combination, on the recovery of rabbit and human erythrocytes frozen at temperatures between -68° and -78° C. It has been found that the addition of sugars prolongs the safe period of exposure to temperatures in the critical range (-3° to -40° C.), thus exerting a protective effect on the red cells of whole blood with acid citrate dextrose. With the use of sugars and rapid freezing and thawing 95 per cent ± 3 of the red cell population may be recovered intact.

TAMMANN and Buchner observed in 1935[1] that certain sugars considerably retarded formation of crystals in an aqueous solution. Evidence has been presented[2] that blood can be frozen and thawed at –3° C. without hemolysis of erythrocytes and that sugars exert a favorable effect on the preservation of erythrocytes stored at temperatures between 0° and –3° C.[3]

Under ordinary conditions, freezing of erythrocytes at temperatures below –3° C., followed by thawing, results in hemolysis. Two procedures have been proposed to avoid or reduce hemolysis. The first one, pioneered by Luyet and co-workers[4] consists of extremely rapid freezing with resulting "vitrification" of water. The second method, introduced by Polge and collaborators[5] and Smith[6] relies on modification of freezing by the addition of glycerol, which permits slow freezing, storage, and thawing of blood with a high rate of recovery of erythrocytes.

The present report deals with the effects observed in vitro of dextrose and lactose on rabbit and human erythrocytes frozen at temperatures between –68° and –78° C. obtained with dry ice and ethanol.

PROCEDURE

Modification of erythrocytes in the present study has been obtained with varying concentrations of dextrose and lactose, alone or in combination. The final concentration of sugars in the liquid medium varied from 0.09 to 1.7M. In all instances, except as noted, the modification consisted of mixing equal parts of acid citrated blood with a sugar solution.

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Freezing has been carried out uniformly at temperatures between -68° and -78° C., using a mixture of ethanol-CO_2 ice. Thawing has been carried out by immersion in water at 37° to 40° C.

The containers have varied considerably in shape and capacity. In most instances they consisted of flat boxes, made of aluminum, from 5 to 15 thousandths of an inch in thickness (0.127 to 0.381 mm.), or made of thin, hard-drawn copper, electroplated with a layer of pure tin. All boxes measured inside 3 to 4 mm. in thickness, but varied considerably in capacity, up to 75 ml. (Fig. 1).

Fig. 1.

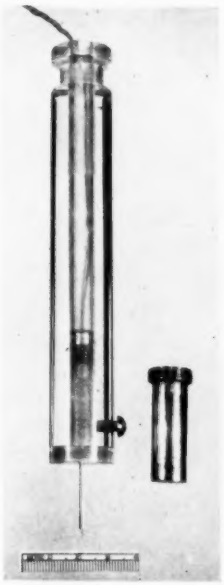

Fig. 2.

Fig. 1.—Flat, thin aluminum or copper container used for freezing and storage of blood.
Fig. 2.—Cylindrical copper container, 11.8 mm. inside diameter and thermocouple holder.

For purpose of more exact thermometric observations and reproducibility, exact geometry of containers is necessary. This requirement is satisfied by using cylindrical containers of thin copper with 11.8 mm. internal diameter. The amount of blood frozen has been between 1.0 and 2.6 ml. (Fig. 2). All thermometric measurements have been carried out with small copper-constantan thermocouples, placed in the center of the mass being frozen, and a graphic recorder.

The isotonic, low dextrose acid citrate dextrose solution used for collection of blood is as follows: trisodium citrate, dihydrate, 2.55 Gm.; citric acid, monohydrate, 0.88 Gm.; dextrose, anhydrous, 1.20 Gm.; pyrogen free distilled water to 100 ml.

The pH of this solution, after sterilization, is 5.0; the amount used for 500 ml. of blood is 63 ml. The acid citrate dextrose solution known as NIH formula A has also been used for the purpose of comparison, with similar results.

The length of modification prior to freezing was either ½ hour or 4½ hours. In all instances thawing was carried out within 15 minutes after completion of freezing and cooling to -68° to -78° C.

RESULTS

Effect of Sugars.—The results of freezing and thawing of erythrocytes will be expressed as "recovery." By recovery is meant the amount of nonhemolyzed erythrocytes, expressed in per cent, which remain after freezing and thawing. The measure of hemolysis is obtained by measure of the total hemoglobin of the sample as compared with the hemoglobin of the supernatant obtained by centrifugation after freezing and thawing. The data on recovery of blood of rabbits and humans, frozen and thawed, are similar. The results reported here are based on experiments carried out on human material, except as otherwise noted.

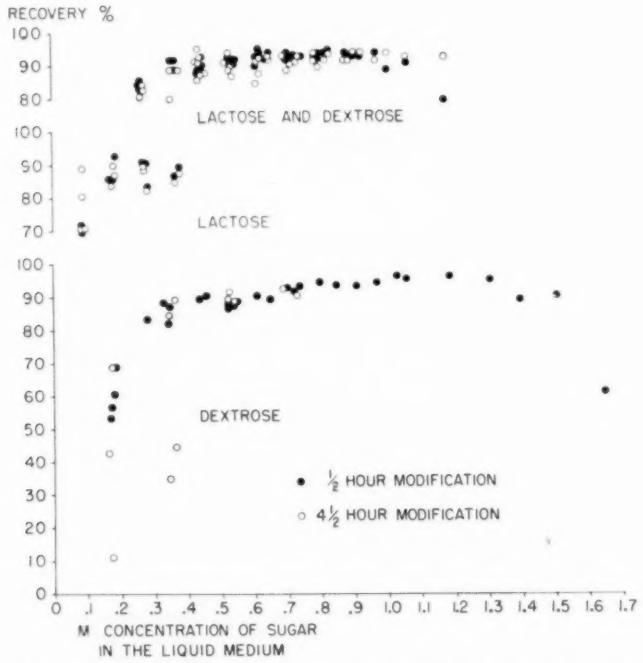

Fig. 3.—Effect of the nature and concentration of sugars and the length of modification prior to freezing on the recovery rate of erythrocytes after freezing and thawing. Blood with acid citrate dextrose is mixed with equal volumes of the sugar solution to obtain in the supernatant fluid the molarity noted on the abscissa.

The recovery of erythrocytes of human acid citrated blood, frozen without agitation in the flat metal boxes, averages 29 per cent. The effect of the concentration and nature of sugars on the recovery rate of frozen human erythrocytes is shown in Fig. 3. In all instances blood collected in the isotonic, low dextrose acid citrate dextrose solution was mixed in equal parts with solutions of lactose and dextrose of such concentration as to obtain the final molarity in the supernatant shown on the abscissa, and was frozen in the same flat metal boxes.

Lactose has an optimal protective effect in a concentration of about 0.2M, dextrose in a concentration between 1.0 and 1.3M, and a mixture of lactose and dextrose in a concentration between 0.62 and 0.97M (Fig. 3). The relative concentration weight per weight of lactose and dextrose has been varied from 1:4 to 4:1; a weight per weight mixture of 3 parts of lactose and 2 of dextrose has given the best results. Under optimal conditions, using 5 to 25 ml. of citrated blood modified by the addition of an equal part of a solution that contained 0.435M lactose and 0.555M dextrose the recovery has averaged 95 per cent ± 3.

The length of modification is not a critical factor, within the experimental limits (½ hour to 4½ hours), except that when dextrose alone is used the optimal length of modification varies with the concentration of sugar.

With rabbit blood, using for modification solutions of lactose and/or dextrose, with a total molarity from 0.4 to 0.7, the recovery rate varied from 79 to 91 per cent with a mean of 86 per cent in 7 specimens of blood frozen and immediately thawed.

Relationship of Mean Corpuscular Volume of Erythrocytes and Recovery.—The relationship between water loss from erythrocytes, expressed as variations of the mean corpuscular volume (MCV), the nature of modifying sugars, the length of modification, and the recovery rate, are shown in Fig. 4. The results suggest that optimal recovery is obtained with a mean corpuscular volume between 60 and 80 cμ.

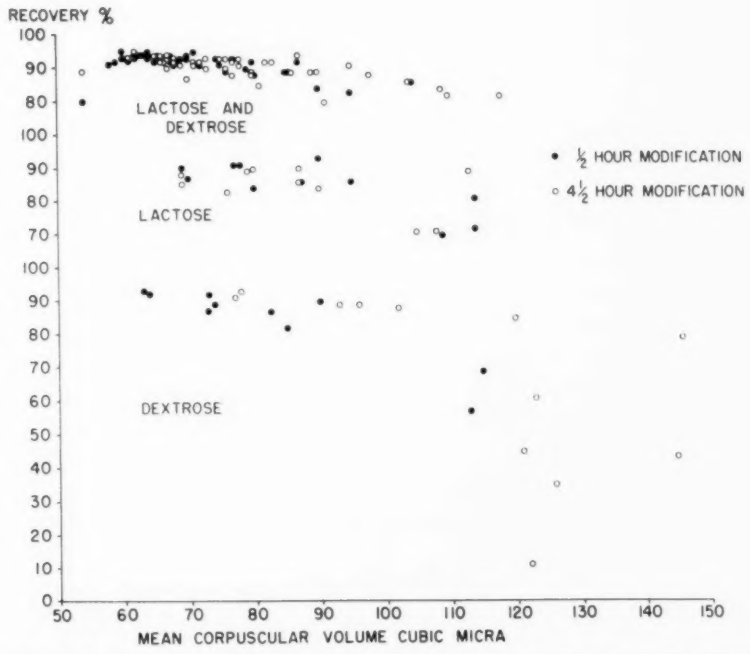

Fig. 4.—Relationship of the nature of sugars, the length of modification, and the recovery of red cells to the water loss, expressed as variation of the mean corpuscular volume. The experimental conditions are the same as represented in Fig. 3.

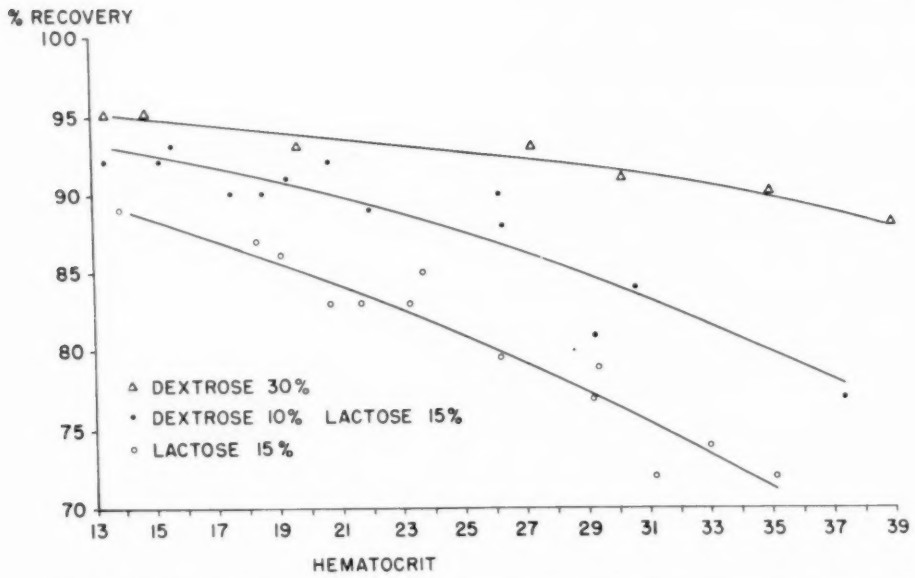

Fig. 5.—Effect of hematocrit on recovery of red cells after they have been frozen and thawed. Variations of the hematocrit value were obtained by removing different amounts of the supernatant, prior to freezing, from the same mixture of acid citrated whole blood and sugars.

Effect of Relative Concentration of Red Cells.—A relationship has been noted between the relative concentration of red blood cells (hematocrit) and the rate of recovery of cells frozen and thawed (Fig. 5). Variations in the hematocrit were obtained by removing a portion of the plasma-sugar mixture after modification and prior to freezing. With increasing relative concentration of the red cell mass, the recovery of red cells after freezing and thawing diminishes. This phenomenon occurs in varying degrees with red cells modified with lactose, dextrose, or a mixture of the two.

Effect of Cooling and Warming Velocities.—When blood is frozen without agitation in the flat metal containers, good results have been obtained when the time of cooling from room temperature to substantially near –60° C. is under 20 seconds. Thawing must be carried out at a similar speed. Freezing or thawing periods of more than 20 seconds result in greater deterioration of the red cells. A typical freezing curve is shown in Fig. 6. The optimal cooling speed in the flat aluminum containers, without motion, has not been determined exactly because of technical difficulties.

The speed of cooling and warming of blood that has been frozen determined with the cylindrical copper containers is considered critical between –3° and –40° C. The –3° C. level has been established by previous observation: blood can be frozen and thawed without appreciable hemolysis (6 mg. per 100 ml. of supernatant plasma) if the temperature of freezing is not lower than –3° C.[2]

The temperature of –40° C. has been suggested by a series of experiments in which blood, modified with equal volumes of 25 per cent dextrose solution,

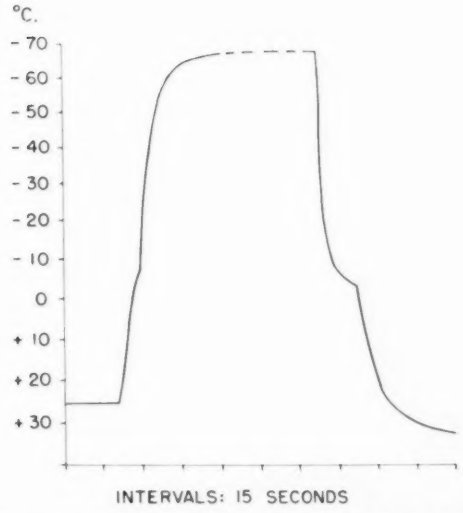

Fig. 6.—Typical cooling and warming curve obtained during freezing of 30 ml. of a mixture of equal parts of blood with acid citrate dextrose and sugar solution in the flat metal containers (Fig. 1) without agitation.

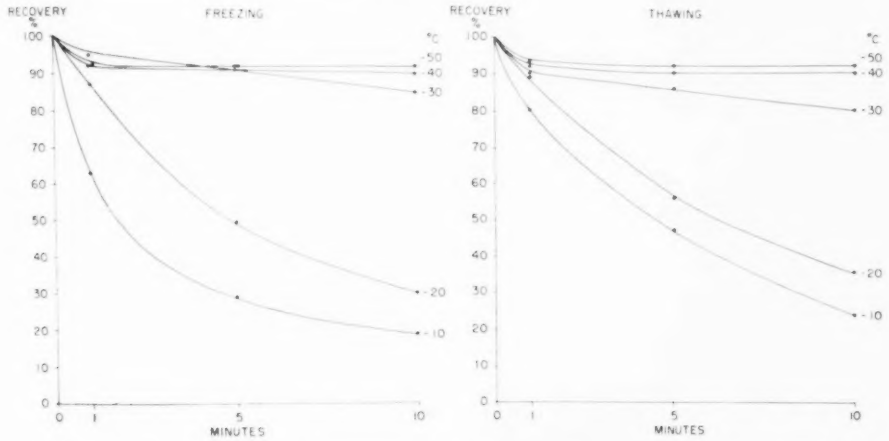

Fig. 7.—Effect of time of exposure at temperatures between –10° and -50° C. during freezing and thawing on the recovery of red cells. The red cells are those of blood with acid citrate dextrose modified by mixing with an equal volume of 25 per cent dextrose solution. Two milliliters of blood-sugar mixtures were tested in the 11.8 mm. cylindrical container (see Fig. 2).

was maintained at a constant temperature for varying periods of time during freezing and thawing (Fig. 7). It will be noted that maintenance of modified blood for one minute during freezing or thawing at a temperature of –10° C. results in a very poor recovery of red cells.

However, permanence at a temperature of –40 C. during either freezing or thawing for periods of as much as 10 minutes is compatible with good recovery. For this reason, cooling and warming velocities between –3° and –40° C. are reported.

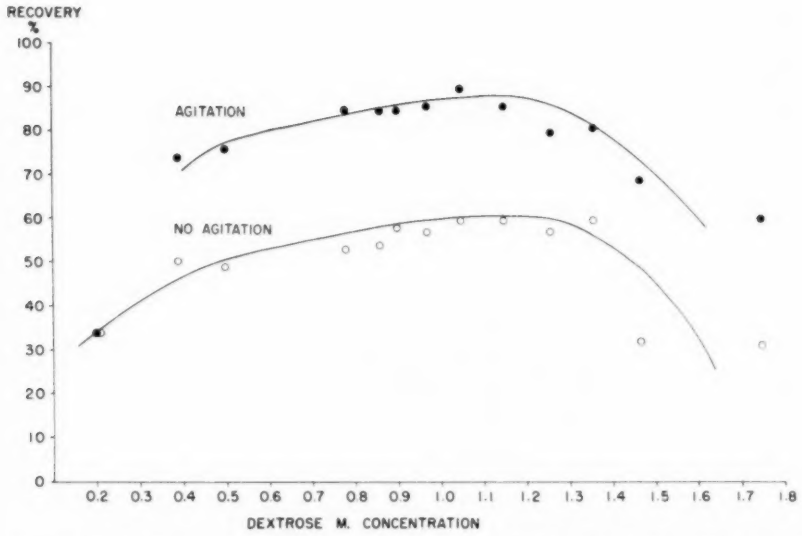

Fig. 8.—The effect of agitation on the recovery of red cells after freezing and thawing with varing concentrations of dextrose. Two milliliters of blood-sugar mixtures were used in the 11.8 mm. cylindrical container (see Fig. 2).

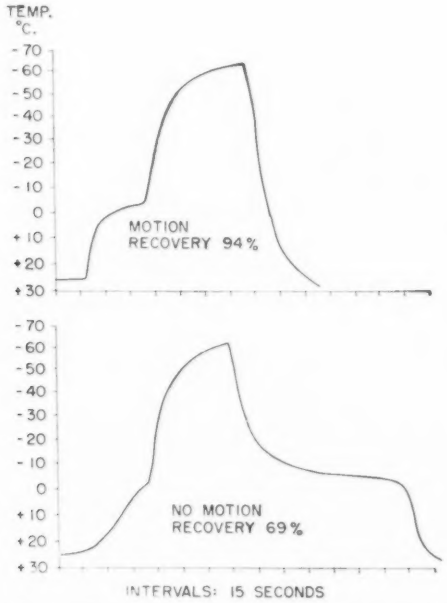

Fig. 9.—Temperature curves during cooling and warming of blood, with and without motion, and recovery of red cells. Acid citrated blood was modified by mixing with equal amounts of 25 per cent dextrose and 2.0 ml. was tested in the 11.8 mm. cylindrical container (see Fig. 2).

Effect of Motion.—It has been noted that agitation of blood during freezing and thawing improves recovery. Although any form of agitation during freezing and thawing of erythrocytes improves the recovery, better results have been obtained with violent to and fro motion or with a vibratory motion at 1,800 to 3,600 c.p.m. The geometry of the container greatly influences the effect of motion. In flat or cylindrical containers with fluid of 3 mm. or less thickness, motion has no effect. Significant effects are noted when the thickness of the fluid is 10 mm. or more.

The effect of agitation is noted over the entire range of concentrations of sugar (Fig. 8). Motion during cooling and warming of red cells results in a much higher rate of recovery than when no motion is applied. Fig. 9 shows the effect of motion on the cooling and warming curves and on the rate of recovery of two identical units of red cells.

Agitation during warming only is more effective than agitation during cooling only. It has also been noted that the warming time is always shorter when cooling is carried out with motion and, correspondingly, the recovery is better (see Table I). This observation is even more significant if one considers that motion slows the cooling time. These observations are not necessarily true when containers of different capacity and configuration are used.

TABLE I. COOLING IN CO_2 ICE–ETHANOL

COOLING	WARMING	RECOVERY (% OF OPTIMAL)
Motion 19	Motion 8	95
No motion 13	Motion 34	86
Motion 19	No motion 52	80
No motion 13	No motion 95	69

The figures noted express in seconds cooling and warming velocities between –3° and –40° C. and represent averages from 5 experiments. Two milliliters of blood was frozen in the 11.8 mm. copper cylinder. In this experiment blood was modified by mixing with equal volumes of 25 per cent dextrose solution.

DISCUSSION

The possible mechanism of the protective effect of sugars on erythrocytes may be discussed in the light of theories on the nature of the damage to red blood cells during freezing and thawing.

In an aqueous solution which is being frozen, other conditions being equal, the size and number of the water crystals is dependent on the velocity of cooling: faster cooling produces smaller and more numerous crystals. If we accept for the moment the view that damage to cells being frozen and thawed is due, in part at least, to mechanical phenomena of pressure and transfixion, it follows that slower cooling and warming velocities should cause greater cellular disruption. The dangerous temperature range, as defined by Luyet and Hartung,[7] is from near zero to about –50° C., and a few milliseconds only are required to

kill living organisms at these temperatures. By ultra rapid freezing and thawing in warm saline, Luyet recovered about 75 per cent of oxalated ox erythrocytes.[8]

It has been shown with the use of electronmicroscopy of thin films of plasma, frozen at the temperature of liquid nitrogen, that no matter how rapidly freezing takes place, water crystals do form.[9] Ultra rapid freezing of various types of cells as employed by Luyet and the term "vitrification" used in this connection must be regarded, in the light of these observations, as indicating the formation of ice crystals of minimal size, incapable of disrupting the cellular structure.

When freezing is rapid, small crystals are formed uniformly throughout the mass being frozen, i.e., intracellularly and extracellularly. When the fluid medium freezes slowly, large extracellular crystals are formed, which results in the removal and immobilization of pure water. In effect, this causes the formation of an increasingly hypertonic medium, with dehydrating effect on the cells. The delicate lipoproteinic structure of the red cell surface is thus exposed to a disrupting effect, as shown by Lovelock.[10] The same mechanism of damage applies during thawing.

The salt concentration, at which this damage occurs, is of some interest. When oxalated ox blood is frozen at –3° C. approximately 81.2 per cent of water is separated in solid form.[11] It has been shown that acid citrated human blood may be solidified at a temperature of –3° C. Under these conditions, upon standing, the red cells settle, which indicates that they are freely floating in the remaining water, exposed to a concentration about 0.75M of sodium chloride. Blood thus solidified may be kept at –3° C. without excessive deterioration of the red cells for several weeks.[2] Lovelock has reported that with a concentration of sodium chloride 0.85M or greater, there is a sharp increase in the cholesterol and phospholipids in the suspending medium of red blood cells, which indicates damage to the cell membrane from exposure to high ionic strength.[12] It may be readily concluded that when sodium chloride rises to a concentration between 0.75 and 0.85M severe damage to the cell membrane occurs on prolonged contact which leads to hemolysis. Undoubtedly this phenomenon plays a major part in the damage to red cells under conditions of relatively slow freezing of blood at temperatures below –3° C.

Formation of large extracellular crystals and, therefore, the exposure of red cells to excessive ionic concentration can be avoided by very rapid freezing of the blood. An entirely different method of protecting red cells by slowing the formation of ice crystals in blood that is being frozen is that based on the observation of Tammann and Buchner[1] and Lusena.[13] These investigators have shown that certain alcohols, glycols, sugars, and proteins slow the rate of water crystal growth in a solution. The mechanism of this action is not definitely known, but is probably related to the property of these components to be strong hydrogen bonders, and consequently to bind water. This property prevents or reduces the damage that results from exposure of the cellular elements to excessively high ionic concentration during slow freezing.

Polge and associates[5] found that fowl spermatozoa suspended in 15 to 20 per cent glycerol solution in Ringer's solution can be frozen slowly at –79° C. and resume full motility after thawing. The method was applied by Smith[6] to rabbit and human erythrocytes.

In the method of freezing erythrocytes presented in this article we have combined the protective effect of sugars to that of rapid freezing. In this manner we have succeeded in lengthening the critical time of exposure of red cells to freezing temperatures from a few milliseconds to 15 or more seconds, thus rendering the method practical for substantially large amounts of blood.

We have experimentally shown that a mixture of lactose and dextrose produces a better recovery of red cells after freezing and thawing than either sugar when used alone. It is presumed that this effect is the product of several mechanisms: (1) lactose, which does not penetrate the cells, binds extracellular water, which slows the formation of extracellular crystals, until with supercooling rapid homogeneous nucleation leads to the formation of numerous small crystals; (2) at the same time, by its osmotic effect, lactose produces a dehydration of the cells. This, if not excessive, increases the concentration of intracellular proteins, thus increasing the water binding capacity within the cells. Cellular elements poor in water, such as epithelial elements of the skin, withstand relatively slow freezing without apparent damage.[14] Some protective effect of sugars on red cells is noted even without intracellular water loss (Fig. 4). (3) The high concentration of extracellular dextrose acts in a way similar to that of lactose, but in addition it penetrates the cell membrane. In the intracellular position it assists in binding water and slowing the rate of formation of ice crystals.

Ordinarily, dextrose that enters red cells carries a certain amount of water, with consequent swelling of the red cells. Under the experimental conditions the amount of water that enters the cells is limited by the extracellular pull of the high concentration of lactose and dextrose. Thus, the lactose-dextrose mixture acts as an osmotic buffer on the red cells.

It has been shown that with increasing relative concentration of the erythrocytes the recovery of erythrocytes after freezing and thawing diminishes. Steinbuch and Quentin[15] made a similar observation on red cells frozen and thawed with the aid of macromolecular substances such as dextran. We consider this phenomenon as being one of coincidence, caused by crystal formation and growth. For each crystal being formed the number of red cells involved, and possibly damaged, is proportional to the density of the population.

SUMMARY

Addition of a solution of lactose, dextrose, or a combination of the two sugars prolongs the safe period of exposure to temperatures in the critical range (–3° to –40°), thus exerting a protective effect on red cells of whole blood with acid citrate dextrose. Under optimal conditions, when a mixture of lactose and dextrose is used, 95 per cent ± 3 of the red cell population is recovered intact after freezing and thawing.

REFERENCES

1. Tammann, B., and Buchner, A.: The Undercooling Properties of Water and the Linear Velocity of Crystallization of Ice in Aqueous Solution, Ztschr. anorg. u. allg. Chem. **222**: 371, 1935.
2. Strumia, M. M.: Freezing of Whole Blood, Science **110**: 398, 1949.
3. Strumia, M. M., McGraw, J. J., Dolan, M., and Colwell, L. S.: Effect of Sugars on Erythrocytes Preserved at 0° to −3° C., Proc. Soc. Exper. Biol. & Med. **75**: 675, 1950.
4. a. Luyet, B. J.: The Vitrification of Organic Colloids and of Protoplasm, Biodynamica **1**: No. 29, 1937.
   b. Luyet, B. J., and Gehenio, P. M.: Life and Death at Low Temperatures, Biodynamica, Normandy, Mo., 1940 (Biodynamica monographs on general physiology, No. 1), (Reprinted from Biodynamica, No. 33, 1938, No. 48, 1939, No. 60, 1940).
5. Polge, C., Smith, A. U., and Parkes, A. S.: Revival of Spermatozoa After Vitrification and Dehydration at Low Temperatures, Nature, London **164**: 666, 1949.
6. Smith, A. U.: Prevention of Haemolysis During Freezing and Thawing of Red Blood Cells, Lancet **2**: 910, 1950.
7. Luyet, B. J., and Hartung, M. C.: Death by Devitrification in the Nematode Anguillula Aceti, Biodynamica **3**: 363, 1941.
8. Luyet, B. J.: Effects of Ultra-rapid and of Slow Freezing and Thawing on Mammalian Erythrocytes, Biodynamica **3**: 363, 1941.
9. Meryman, H. T., and Kafig, E.: The Study of Frozen Specimens, Ice Crystals and Ice Crystals Growth by Electron Microscopy, Bethesda, Md., 1955, Naval Medical Research Institute, vol. 13, p. 529.
10. Lovelock, J. E.: The Denaturation of Lipid-Protein Complexes as a Cause of Damage by Freezing, Proc. Roy. Soc., London, s. B **147**: 427, 1957.
11. Luyet, B., and Schmidt, P.: Determination of Amount of Ice Formed in Blood at Freezing Temperatures, Fed. Proc. **9**: 81, 1950.
12. Lovelock, J. E.: The Haemolysis of Human Red Blood-Cells by Freezing and Thawing, Biochim. et biophys. acta **10**: 414, 1953.
13. Lusena, C. V.: Ice Propation in Systems of Biological Interest. III. Effects of Solutes on Nucleation and Growth of Ice Crystals, Arch. Biochem. **57**: 277, 1955.
14. Strumia, M. M., and Hodge, C. C.: Frozen Human Skin Grafts, Ann. Surg. **121**: 850, 1945.
15. Steinbuch, M., and Quentin, M.: Reserches sur la congelation du globule rouge en milieu macromoleculaire, Vox Sang. **3**: 123, 1958.

THE PRESERVATION OF BLOOD FOR TRANSFUSION

V. Posttransfusion Survival of Red Cells Modified with Sugars, Frozen, and Stored in the Frozen State

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Dextrose and lactose improve considerably the in vitro recovery of erythrocytes subjected to rapid freezing and thawing. This report deals with the effect of freezing and thawing of erythrocytes modified by a mixture of dextrose and lactose on the posttransfusion survival measured with Cr^{51} tagging.

It will be shown that the 24 hour posttransfusion survival of Cr^{51} radioactivity of red cells frozen and stored in the frozen state at −93° C. ± 2 remains unchanged for at least 721 days, and represents an average of 77 per cent of the original amount of the red cells frozen.

In a previous article[1] it has been shown that dextrose and lactose improve considerably the recovery in vitro of erythrocytes subjected to rapid freezing and thawing.

In the present report are presented the results of posttransfusion survival of 10 units of rabbit erythrocytes and 38 units of human erythrocytes modified by a mixture of dextrose and lactose, frozen, and stored in the frozen state for varying periods of time.

PROCEDURE

The amount of rabbit blood frozen and stored has varied from 7 to 20 ml., and of human blood from 20 to 50 ml. The storage temperatures in the range of −58° and −93° C. were obtained with the use of sub-zero equipment.* Storage temperature of about −70° C. was obtained by use of dry ice.

The amount of blood transfused has been 2 ml. in the rabbit and 10 ml. in the human.

Methods for the measurement of blood volume and for survival studies by tagging with Cr^{51} have been presented.[2, 3]

All the studies of posttransfusion survival in humans have been carried out with red cells of whole, acid citrated blood frozen in thin flat metal containers, at a temperature of −68° to −78° C. obtained with dry ice–ethanol, after modification by addition of equal parts of the following solution: dextrose, anhydrous, 10 Gm.; lactose, monohydrate, 15 Gm.; distilled water to 100 ml., 0.99M.

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RESULTS

The effects of freezing and thawing of erythrocytes in vitro have been expressed as "recovery." The results of in vivo studies will be expressed as "survival." Survival indicates the amount of radioactivity of frozen, stored, thawed, and transfused erythrocytes that remain in circulation 24 hours after transfusion, as determined with the Cr^{51} technique. The values reported as survival include all losses, that is those occurring in vitro and those occurring in vivo.

Per cent recovery in vitro times per cent surviving radioactivity 24 hours after transfusion divided by 100 equals percentage survival.

A slight loss of erythrocytes occurs in the period immediately after thawing (within one hour). This loss varies with the nature and concentration of sugar used for modification. With the lactose-dextrose mixture used in the experiments reported here the loss amounts to less than 3 per cent up to 3 hours after thawing.

Erythrocytes have been transfused within one hour of thawing. After thawing the erythrocytes are not subjected to any further preparation, except for the occasional removal of some of the plasma-sugar mixture.

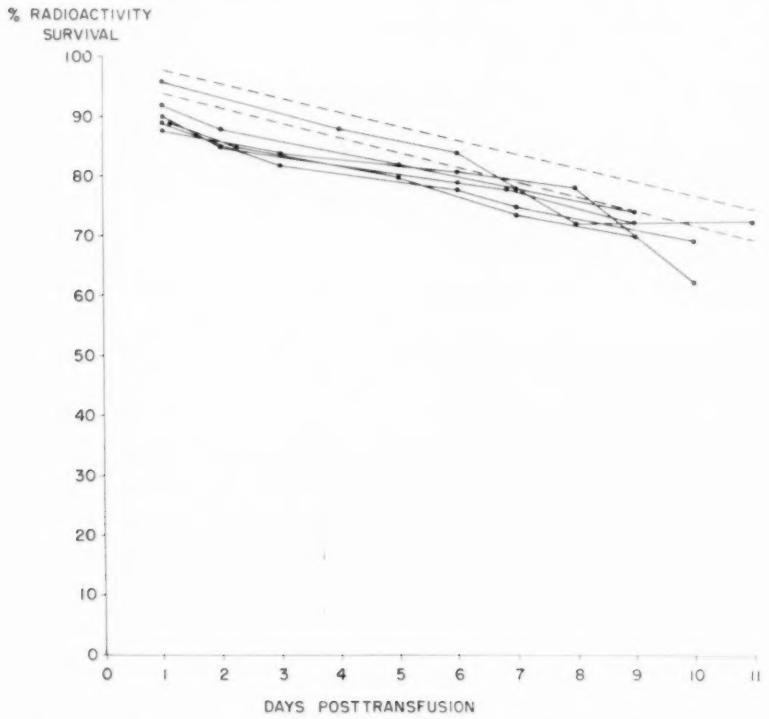

Fig. 1.—Rate of decline of Cr^{51} radioactivity of 6 units of blood, modified with lactose-dextrose mixture, frozen, and thawed. Four units were autotransfused, and 2 were transfused into compatible recipients. The interrupted lines represent the survival of 38 units of autotransfused fresh blood.

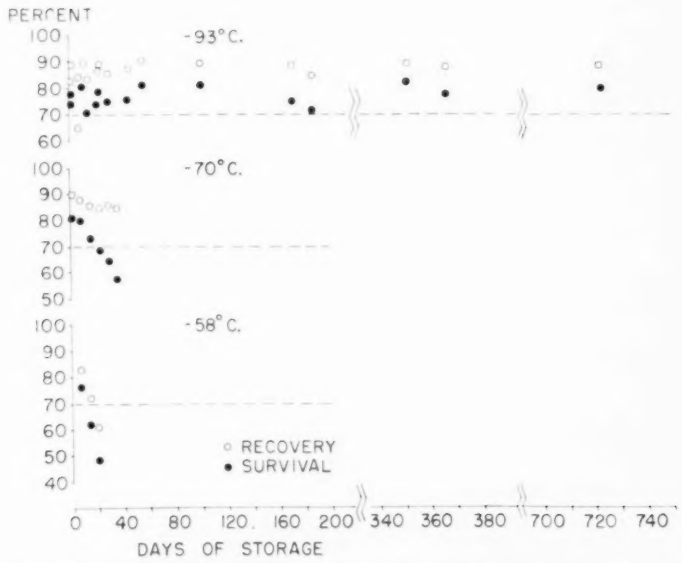

Fig. 2.—Effect of temperature of storage and length of storage on recovery and post-transfusion survival of Cr^{51} radioactivity of red cells modified with lactose-dextrose mixture before being frozen. The figures given for the survival include all losses, in vitro and in vivo.

Survival of Rabbit Erythrocytes.—For modification of rabbit blood used for transfusion, lactose, dextrose, and lactose-dextrose solutions were used. When used alone for modification of rabbits' blood, lactose gave an optimal posttransfusion survival, whereas glucose alone gave a survival slightly below the average obtained with the lactose-dextrose mixture. The average 24 hour radioactivity survival of erythrocytes frozen, thawed, tagged with Cr^{51}, and immediately transfused in seven rabbits was 97 per cent of the average obtained after transfusion of fresh citrated blood.

Two rabbits were similarly transfused with erythrocytes frozen after modification with sugars, and stored for one week at –60° C. The radioactivity of erythrocytes 24 hours after injection averaged 97 per cent of that obtained from transfusion of fresh whole blood.

Survival of Human Erythrocytes.—Of 38 transfusions of frozen human erythrocytes some were autotransfused and some were transfusions in compatible recipients.

The survival of six different units of human erythrocytes frozen, thawed, stored for one day or less, and transfused within one hour of thawing is shown in Fig. 1. The values are slightly below the optimal established for 38 units of normal red cells autotransfused within 2 hours of collection, but the daily rate of decline is similar (2.17 per cent per day ± 0.26). No difference was noted between 4 autotransfusions and 2 transfusions into compatible recipients.

In Fig. 2 are shown the effects of the temperature of storage on the recovery and survival of frozen red cells. In all the experiments the temperature of freezing and the modification by addition of sugars in equal parts of the

TABLE I

RECIPIENTS	LENGTH OF STORAGE	(% OF RADIOACTIVITY) DAYS POSTTRANSFUSION											LOSS PER DAY
		1	2	3	4	5	6	7	8	9	10	11	
N. B.	1 hr.	92	88			82		78		74			2.25
J. P.	1 hr.	96			88		84		72			72	2.40
J. N.	1 hr.	89		82			78	75			69		2.22
L. K.	1 hr.	90	85				79	78		72			2.25
J. B.	1 Day	89	85			80		74		70			2.38
M. Mc.	1 Day	88		84			81		78		62		2.89
R. C.	7 Days	76	72			66		65		63			1.63
C. A.	9 Days	90	92			80		82		81			1.13
R. B.	14 Days	84		77		74		71		68			2.00
R. Ba.	21 Days	85		82		78			74	73			1.50
J. H.	22 Days	89		87			82		79		72		1.89
T. N.	29 Days	87	84			74		69		65			2.75
W. G.	44 Days	86			79		74		71			67	1.90
G. O.	55 Days	90	92					82		81	71		2.11
R. L.	99 Days	91	88			80	80			75			2.00
R. Z.	169 Days	83		74			67		60		57		2.89
R. R.	184 Days	84			75		69				56		3.11
R. S.	351 Days	92			89		84		79		76		1.78
W. B.	366 Days	84		81		77		75		72			1.50
J. M.	721 Days	90		88		85		80		75			1.88

Average 24 hour posttransfusion radioactivity survival of frozen Cr^{51}-tagged red cells 88 per cent ± 4.
Average daily loss of radioactivity of frozen Cr^{51}-tagged red cells second to the eleventh day 2.12 per cent ± 0.50.
The 100 per cent in this table is the amount of red cells recovered after freezing and thawing.
The "loss per day" represents the average daily loss from the second day to the end of observation.

lactose-dextrose mixture are comparable. It can be readily seen that with a storage temperature of –58° C. there is rapid deterioration of the rate of recovery and rate of survival of erythrocytes. With storage at –70° C. the rate of recovery is fairly well maintained but the survival deteriorates more rapidly. At –93° C. ± 2 both recovery and survival remain at an essentially even level for a period of at least 721 days. (See Table I.)

In all survival studies the blood was frozen within 24 hours of collection. Blood frozen after several days of storage under usual condition of refrigeration has shown good in vitro recovery after thawing.

In the present series of experiments the survival of radioactivity of all transfused erythrocytes, which have been frozen, thawed, and stored at –93° C. for periods up to 721 days, including all losses in vitro and in vivo, has averaged 77 per cent ± 0.5. This survival may be compared with the optimal survival of radioactivity of fresh autotransfused Cr^{51}-tagged red cells 24 hours after transfusion, which averages 94 per cent ± 2. The survival value of frozen cells may also be compared with the survival of radioactivity of red cells stored for 21 days in acid-citrate-dextrose solution at temperatures between 1 and 5° C., which varies between 70 and 84 per cent, depending on the nature of the receptacle, the temperature of storage, and other conditions.

DISCUSSION

The effect of storage on the frozen erythrocytes is due to the well-known fact that ice crystals are not of a static nature, but tend to grow, a phenomenon also referred to as recrystallization. The growth rate is high at temperatures near the melting point; however, crystal growth occurs at much lower temperatures. Thus, at –70° C. crystals 1 μ long are formed in a previously noncrystalline ice film in 30 seconds.[4] We have shown that with storage at temperatures between –58° and –70° C. deterioration of red cells occurs in a few weeks (see Fig. 2). This is probably due to the growth of ice crystals. Crystal growth ceases at a temperature of about –130° C.[5, 6]; from our experimental work the bulk of red cells, properly frozen, survives at a temperature of –93° C. ± 2 for a period of at least 2 years.

Although much of the damage from slow freezing is undoubtedly due to the exposure of cellular elements to a high ionic concentration, there is evidence that mechanical effect of water crystals by crushing and transfixion accounts in part at least for the loss of red cells during storage in the frozen state at temperatures that permit growth of ice crystals. This contention is supported by the experimental data which show that with increase of the relative concentration of red cells there is a corresponding increase of the loss of cells during freezing.[1]

It has been shown that modification of red cells with either dextrose, lactose or a combination of the two protects the red cells from the effects of freezing and thawing. However, the posttransfusion survival of modified cells does not necessarily follow the recovery of red cells in vitro.

Whole blood with acid citrate dextrose treated with equal parts of 15 per cent dextrose, with a resulting final molarity in the supernatant fluid of 0.55, frozen, and thawed, shows a recovery of 92 per cent, but upon transfusion the bulk of red cells is immediately destroyed. Whole blood with acid citrate dextrose, treated with 25 per cent dextrose solution in equal parts, with a resulting final molarity in the supernatant fluid of 0.81, frozen, and thawed, shows a recovery of 93 per cent of red cells but the posttransfusion survival is likewise very poor. Penetration of dextrose into the red cells is probably responsible for this phenomenon.

The study of the daily loss of transfused red cells, as shown in Table I, clearly indicates that the rate of decline of transfused cells is not related to the length of storage. The average daily decline from the second to the ninth or eleventh day is 2.12 per cent ± 0.50, data similar to those obtained with normal fresh autotransfused cells (daily loss 2.17 per cent ± 0.26). Much greater variation is noted in the first 24 hour loss. Normally this loss is 6 per cent ± 2. In the transfusion of frozen red cells the loss averages 12 per cent ± 4.

We interpret this greater loss and the greater differences between observations as being due to variations in the technique and, more exactly, to variation in the thickness of the mass of blood being frozen. Cells damaged by improper technique of freezing or thawing are immediately removed from circulation. Cells which have not been damaged in the process of freezing and thawing, however, survive normally.

Protection of cellular elements by ultra rapid freezing, applied previously to thin films of blood, was more recently used by Meryman and Kafig[7] by spraying blood directly into liquid nitrogen, with the formation of small spheres of frozen material, ranging up to 0.9 mm. in diameter. Only two posttransfusion survival studies were done in human beings. Six and 7 per cent of the red cells, respectively, were lost through hemolysis prior to transfusion; the 24 hour posttransfusion survival of radioactivity of Cr^{51}-tagged cells was 86 and 82 per cent, respectively. The radioactivity loss of the red cells in the period subsequent to the first day was 2.6 and 4 per cent per day.

At the practical level, the most important problem is the development of a container of capacity sufficient for a whole unit of blood and of such design and material to permit very rapid heat exchange. Use of lower temperature coolant than the ethanol–Co_2 ice mixtures, and a means of keeping the coolant in active circulation may be expected to improve both recovery and survival of red cells.

SUMMARY

Human red cells, frozen with the addition of a lactose-dextrose mixture, and thawed show a posttransfusion survival, measured as Cr^{51} radioactivity similar to that of fresh red cells except for an increased first day loss of 12 per cent ± 4, as compared with fresh blood 6 per cent ± 2. Rabbit erythrocytes behave in a similar way.

The 24 hour posttransfusion survival of Cr^{51} radioactivity of red cells frozen and stored in the frozen state at -93° C. ± 2 remains unchanged for at least 721 days, and represents an average of 77 per cent of the original amount of red cells frozen.

REFERENCES

1. Strumia, M. M., Colwell, A. B., and Strumia, P. V.: The Preservation of Blood for Transfusion. IV. In Vitro Recovery After Freezing and Thawing of Red Cells Modified With Sugars, J. Lab. & Clin. Med. **56**: 576, 1960.
2. Strumia, M. M., Taylor, L., Sample, A. B., Colwell, L. S., and Dugan, A.: Uses and Limitations of Survival Studies of Erythrocytes Tagged With Cr^{51}, Blood **10**: 429, 1955.
3. Strumia, M. M., Colwell, L. S., and Dugan, A.: The Erythropoiesis in Anemias. I. The Mixing Time and the Immediate Posttransfusion Disappearance of T-1824 Dye and of Cr^{51} Tagged Erythrocytes in Relation to Blood Volume Measurements, Blood **8**: 128, 1958.
4. Meryman, H. T., and Kafig, E.: Naval Medical Research Institute Report Project NM 000 018.01.09, Bethesda, Md., 1955.
5. Pryde, J. A., and Jones, G. O.: Properties of Vitreous Water, Nature, London **170**: 685, 1952.
6. Burton, E. F., and Oliver, W. F.: The Crystal Structure of Ice at Low Temperatures, Proc. Roy. Soc. London, S. A. **153**: 166, 1936.
7. Meryman, H. T., and Kafig, E.: Rapid Freezing and Thawing of Whole Blood, Proc. Soc. Exper. Biol. & Med. **90**: 587, 1955.

SERUM COMPLEMENT AND BACTERIOPHAGE NEUTRALIZATION TITERS IN HUMAN INFECTIONS, LEUKEMIAS, AND LYMPHOMAS

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Serum bacteriophage neutralizing activity (PhN) was studied in 82 normal subjects, 103 individuals with infections, 39 patients with leukemias and 47 patients with lymphoma or sarcoma. The normal mean titer was 10 PhN_{50} units per milliliter. Low titers (< 2 units per milliliter) were found in most acute infections, chronic lymphocytic leukemia, active Hodgkin's disease, and some cases of reticulum cell and lymphosarcoma. In infections a two- to ninefold increase in titer occurred within 48 hours during recovery. Normal or high PhN titers were found in patients with chronic infections, myelogenous and monocytic leukemia, inactive Hodgkin's disease, and most cases of reticulum cell and lymphosarcoma.

Serum complement titers were studied in 82 normal subjects, 42 subjects with infections, 28 patients with leukemia, and 25 patients with lymphoma or sarcoma. The normal mean titer was 31.4 $C'H_{50}$ units per milliliter. Normal or low titers were found in most acute infections. A two- to threefold increase occurred usually within 48 hours during recovery. Normal or high titers occurred in most cases of chronic infection, leukemia, Hodgkin's disease and sarcoma.

It is suggested that: (1) Serum PhN activity may reflect properdin; (2) Complement and properdin may participate in host resistance to infection.

THE recognition of a new serum substance, properdin, by Pillemer and co-workers[1] renewed interest in the study of natural host resistance to infection. In 1956, Van Vunakis and co-workers[2-4] provided evidence that T_2r+ *Escherichia coli* bacteriophage neutralization activity of fresh serum may reflect properdin levels. They indicated that this activity was demonstrable only in the presence of all four components of complement ($C'_{1, 2, 3, 4}$), divalent cations (Mg^{++}), and properdin (P). Serum which contained no properdin (RP) failed to show phage neutralization, whereas the addition of purified properdin to RP restored this activity.

The purpose of this paper is to present data concerning total complement and bacteriophage (*E. coli* T_2r+) neutralizing activity of serum from normal subjects and patients with infections, leukemias, and lymphomas.

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MATERIALS AND METHODS

A. *Serum* Preparation.*—Ten milliliters of blood was drawn aseptically, allowed to clot at room temperature for 20 to 40 minutes, and placed at 2° C. for 2 to 4 hours. After centrifugation at 2,200 g for 6 to 8 minutes, the serum was removed and stored at −22° C. for one to four weeks before testing.

B. *Phage Neutralizing Activity.*—Phage neutralizing activity, hereafter designated as PhN, is standardized in terms of 50 per cent bacteriophage neutralization units PhN_{50} per milliliter of serum. The determinations were performed using the technique described by Barlow, and co-workers.[3, 4] T_2r+ bacteriophage active on *E. coli* B and cultures of *E. coli* B were provided by the same authors.

C. *Complement.*—(C′ is standardized in terms of 50 per cent hemolytic units $C'H_{50}$ per milliliter of serum.) Total complement determinations were performed by means of the methods described by Osler and collaborators.[5] The following five modifications were made: (1) Amboceptor was prepared using the method described by Rapp.[6] (2) Guinea pig complement was prepared from blood taken from 15 normal guinea pigs. The blood was allowed to clot for 20 minutes at room temperature, placed overnight at 4° C., and centrifuged at 700 g for 30 minutes at 0° C. The serum was quickly frozen in a CO_2 ice bath and stored at −22° C. The pooled serum complement titer was 240 $C'H_{50}$ per milliliter. (3) Sheep blood was collected in an equal volume of 2.5 per cent sodium citrate and Merthiolate at a final concentration of 1:10,000. The red blood cells were washed three times with isotonic Veronal buffer which contained 0.1 per cent gelatin. A 5 per cent suspension of the washed cells was prepared by adding 1 ml. of packed cells to 19 ml. of gelatin Veronal buffer. One milliliter of this suspension was lysed with 14 ml. of 0.1 per cent sodium carbonate. The optical density (O.D.) of the clear lysate was determined to be 0.680 by means of a Beckman DU spectrophotometer at a wavelength of 541 mμ. This reading corresponded to 1×10^9 red blood cells per milliliter. (4) Sheep red blood cells were sensitized with an optimal dilution of amboceptor for 15 minutes in a 37° C. water bath. (5) Each complement titration was performed in duplicate. Different aliquotes of diluted test serum were placed in tubes and Veronal buffer was added to bring the total volume in each tube to 6.5 ml. One milliliter of sensitized sheep red blood cells was added to each tube. After incubation and centrifugation the O.D. of each supernatant was read using a Beckman DU spectrophotometer at 541 mμ; gelatin Veronal buffer was used as a blank. A straight line connecting at least three and usually four or five points in the 25 to 75 per cent hemolytic range was used for the calculation of the 50 per cent hemolysis point of the serum tested.

RESULTS

A. *Normal Subjects.*—

1. *Phage Neutralization Activity:* Serum PhN titers were determined in 82 normal subjects from 20 to 40 years of age. Sixty per cent of the subjects were men and 40 per cent were women. No significant difference in titers was found with respect to age and sex. Serum PhN levels ranged from 1.9 to 31.2 PhN_{50} per milliliter with a mean of 10 PhN_{50} per milliliter (Fig. 1). Titers could not be determined in sera of 4 subjects (4.7 per cent) because of the presence of a heat stable factor.† Repeated determination of PhN activity on different aliquots of the same subject's serum revealed that the concentration varied less than 1.5 PhN_{50} per milliliter and remained stable at −22° C. for at least 8 weeks. Five normal subjects were bled weekly for 5 weeks. The PhN titers done on the day after each bleeding varied less than 2.5 PhN_{50} per milliliter.

*Sera used in these studies were obtained from patients of the Tumor Clinic and the Medical Services of the Upstate Medical Center Hospitals, and the Syracuse VA Hospital.

†Further testing of these sera was done by means of the complement fixation technique for the presence of antibody to the virus used in the phage assay. No measurable titer of antibody was found with the use of active and inactive virus particles.

1. *Complement:* Serum complement was determined in 82 normal subjects from 20 to 40 years of age. Seventy per cent of the subjects were men and 30 per cent were women. No significant difference in titers was found with respect to age and sex. Serum complement levels ranged from 20 to 48 $C'H_{50}$ per milliliter with a mean of 31.4 $C'H_{50}$ per milliliter (Fig. 1). Repeated determinations of complement on different aliquots of the same subject's serum revealed that the concentration varied less than 2.5 $C'H_{50}$ per milliliter and remained stable at −22° C. for at least 4 weeks. Five normal subjects were bled weekly for 5 weeks. The complement titers done on the day after each bleeding varied less than 4 $C'H_{50}$ per milliliter.

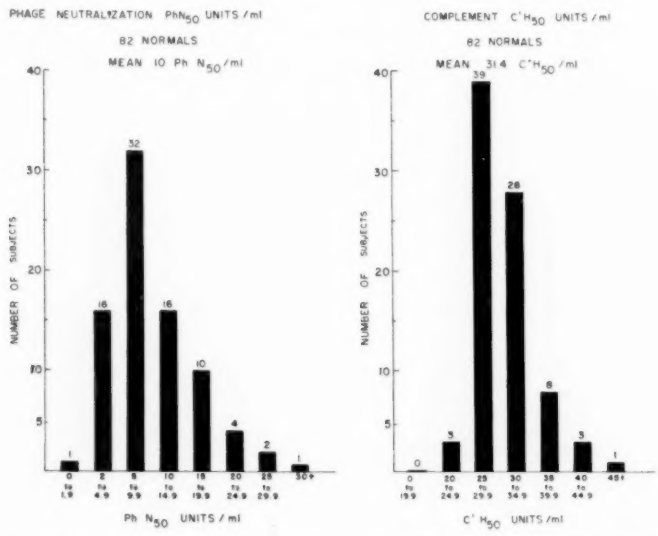
PHAGE NEUTRALIZATION PhN50 UNITS / ml
82 NORMALS
MEAN 10 Ph N50 / ml
NUMBER OF SUBJECTS
Ph N50 UNITS / ml
COMPLEMENT C'H50 UNITS / ml
82 NORMALS
MEAN 31.4 C'H50 / ml
NUMBER OF SUBJECTS
C'H50 UNITS / ml

Fig. 1.—Levels of phage neutralizing activity and complement in serum of normal subjects.

B. *Infections.*—Serum PhN activity was studied in 103 infections in adults (Table I). In 72 of these patients the first blood sample was obtained at the peak of the infection and prior to the institution of therapy. Thirty-five of these acutely ill patients had bacteremia (19 died). In thirty-one patients the first blood sample was obtained 1 to 4 days after the institution of therapy. Seventy-two of the total group of 103 patients had serial determinations of serum PhN performed during the course of the infection. In most patients follow-up blood samples were obtained at 48 and 96 hours, at 7 days, or after 2 to 4 weeks. The total number of PhN determinations made in the entire group of patients with infections was 227; 159 of these were follow-up samples.

The mortality rate in acutely ill patients who had PhN titers of less than 5 PhN_{50} per milliliter was 50 per cent (15 of 30 patients), in patients with titers between 5 and 15 PhN_{50} per milliliter 12 per cent (4 of 34). (Table II).

TABLE I. DISTRIBUTION OF ACUTE INFECTIONS, NUMBER OF PATIENTS STUDIED, AND NUMBER OF SERUM PHAGE NEUTRALIZATION TITERS PERFORMED

INFECTIONS	NO. OF PATIENTS					NO. OF PHAGE NEUTRALIZATION TITERS PERFORMED	
	TOTAL NO. STUDIED	FIRST BLOOD BEFORE TREATMENT		FIRST BLOOD 1 TO 4 DAYS AFTER START OF TREATMENT	NO. OF PATIENTS FOLLOWED	DURING RECOVERY	TOTAL NO.
		SURVIVED	DIED				
Pneumococcal infections (bacteremia in 4)	21	10	3	8	19	41	54
Staph. aureus bacteremia	23	1	12	10	13	36	47
Gram-negative bacteremia	15	7	2	6	9	23	30
Beta hemolytic streptococcus bacteremia	4	2	1	1	3	11	14
Alpha hemolytic streptococcus bacteremia (S.B.E.)	11	8	0	3	11	17	25
Acute lung abscess or empysema	11	10	1	0	7	14	25
Local *Staph. aureus* infections	18	15	0	3	10	17	32
Total	103	53	19	31	72	159	227

There were no fatalities in the seven patients who had titers greater than 15 PhN_{50} per milliliter. Subsequently, titers in 68 of the 72 patients who recovered and whose serum PhN activity was measured periodically during recovery reached levels greater than 5 PhN_{50} per milliliter.

TABLE II. COMPARISON OF SERUM PHAGE NEUTRALIZATION TITERS DURING ACUTE INFECTION AND CONVALESCENCE

INFECTIONS	ACUTE (PhN_{50} UNITS/ML.)						CONVALESCENCE (PhN_{50} UNITS/ML.)		
	< 5		5.1 – 15		> 15		< 5	5.1 – 15	> 15
	NO. OF PATIENTS WHOSE FIRST BLOOD WAS OBTAINED BEFORE TREATMENT						NO. OF PATIENTS WITH HIGHEST TITER DURING CONVALESCENCE OCCURRING IN 2-9 DAYS*		
	SURVIVED	DIED	SURVIVED	DIED	SURVIVED	DIED			
Pneumococcal infections (bacteremia in 4)	5	3	4	0	1	0	2	11	6
Staph. aureus bacteremia	1	8	0	4	0	0	1	7	5
Gram-negative bacteremia	4	2	3	0	0	0	0	3	6
Beta hemolytic streptococcus bacteremia	1	1	1	0	0	0	0	0	3
Alpha hemolytic streptococcus bacteremia (S.B.E.)	0	0	5	0	3	0	0	4	7
Acute lung abscess or empysema	1	1	6	0	2	0	1	3	3
Local *Staph. aureus* infections	3	0	11	0	1	0	0	7	3
Total	15	15	30	4	7	0	4	35	33
Mortality	50%		12%		0%				

*These figures include both "survived" and convalescing patients with first blood obtained before treatment as well as those patients whose first blood was obtained 1 to 4 days after start of treatment.

Serum complement was studied in 42 infections in adults (Table III). In 34 of these patients the first blood sample was obtained at the peak of the illness and prior to the institution of therapy. Nineteen of these acutely ill patients had bacteremia (5 died). In 8 patients the first blood sample was obtained 1 to 4 days after the institution of therapy. Thirty-four of the total group of 42 patients had serial determinations of serum complement performed during the course of infection. In most patients follow-up blood samples were obtained at 48 and 96 hours, at 7 days, or after 2 to 4 weeks. The total number of complement determinations made in the entire group of patients with infections was 106; 72 of these were follow-up samples.

TABLE III. DISTRIBUTION OF ACUTE INFECTIONS, NUMBER OF PATIENTS STUDIED, AND NUMBER OF SERUM COMPLEMENT DETERMINATIONS PERFORMED

INFECTIONS	NO. OF PATIENTS					NO. OF COMPLEMENT DETERMINATIONS	
	TOTAL NO. STUDIED	FIRST BLOOD BEFORE TREATMENT		FIRST BLOOD 1 TO 4 DAYS AFTER START OF TREATMENT	NO. OF PATIENTS FOLLOWED	DURING RECOVERY	TOTAL NO.
		SURVIVED	DIED				
Pneumococcal infections (bacteremia in 4)	10	5	2	3	7	20	27
Staph. aureus bacteremia	9	2	3	4	8	22	27
Gram-negative bacteremia	3	3	0	0	2	3	6
Beta hemolytic streptococcus bacteremia	1	1	0	0	1	3	4
Alpha hemolytic streptococcus bacteremia (S.B.E.)	7	6	0	1	7	10	16
Acute lung abscess or empysema	6	6	0	0	4	6	12
Local *Staph. aureus* infections	6	6	0	0	5	8	14
Total	42	29	5	8	34	72	106

The mortality rate in acutely ill patients who had complement titers less than 29 $C'H_{50}$ per milliliter was 29 per cent (2 of 7), in patients with titers between 30 and 45 $C'H_{50}$ per milliliter 14 per cent (3 of 21). There were no fatalities in the group of patients whose complement titers were greater than 46 $C'H_{50}$ per milliliter. Higher titers of complement occurred during recovery than during acute phase of illness, i.e., levels in 33 of the 34 patients who recovered and whose serum complement was measured periodically during recovery reached levels greater than 30 $C'H_{50}$ per milliliter.

The degree and rapidity of rise of both serum PhN and complement varied during the recovery phase of an acute infection. Examples of serum PhN titers of 4 patients and complement titers of 2 patients with acute pneumococcal infections are shown in Fig. 2. In each patient the first blood sample was obtained at the peak of the infection and prior to therapy. Only the patient who died failed to show a rise in titer. Generally when the acute infection was controlled rapidly with proper therapy and the titer was low at the time of acute illness, the rise in titer was marked and prompt, i.e., two- to ninefold

for PhN and two- to threefold for complement within 48 hours. If the infection was more difficult to control or a normal titer was obtained during the acute phase of illness, the rise in titer was less marked and occurred only after 5 or more days.

C. *Leukemias, Lymphomas, and Sarcomas.*—PhN titers were measured in 39 patients with leukemia and 47 patients with lymphoma or sarcoma. Titers of serum complement were measured in 28 patients with leukemia and 25 patients with lymphoma or sarcoma. The distribution and mean of titers are seen in Figs. 3 and 4.

TABLE IV. COMPARISON OF SERUM COMPLEMENT TITERS DURING ACUTE INFECTION AND CONVALESCENCE

INFECTIONS	ACUTE ($C'h_{50}$ UNITS/ML.) NO. OF PATIENTS WHOSE FIRST BLOOD WAS OBTAINED BEFORE TREATMENT						CONVALESCENCE ($C'h_{50}$ UNITS/ML.) NO. OF PATIENTS WITH HIGHEST TITER DURING CONVALESCENCE OCCURRING IN 2-9 DAYS*		
	< 29		30 – 45		> 46		< 29	30 – 45	> 46
	SURVIVED	DIED	SURVIVED	DIED	SURVIVED	DIED			
Pneumococcal infections (bacteremia in 4)	2	1	3	1	0	0	1	1	5
Staph. aureus bacteremia	0	1	2	2	0	0	0	2	6
Gram-negative bacteremia	1	0	2	0	0	0	0	1	1
Beta hemolytic streptococcus bacteremia	1	0	0	0	0	0	0	1	0
Alpha hemolytic streptococcus bacteremia (S.B.E.)	1	0	4	0	1	0	0	4	3
Acute lung abscess or empysema	0	0	4	0	2	0	0	1	3
Local *Staph. aureus* infections	0	0	3	0	3	0	0	3	2
Total	5	2	18	3	6	0	1	13	20
Mortality	29%		14%		0%				

*These figures include both "survived" and convalescing patients with first blood obtained before treatment as well as those patients whose first blood was obtained 1 to 4 days after start of treatment.

1. *Leukemia:* Serum PhN titers were measured in 28 patients with chronic lymphocytic, 6 patients with myelogenous, and 5 patients with monocytic leukemia. All 28 patients with chronic lymphocytic leukemia had PhN levels less than 8 PhN_{50} per milliliter; 19 of these patients had levels less than 2 PhN_{50} per milliliter. In contrast, patients with other forms of leukemia had normal levels. No evidence of antiphage neutralizing activity was found when normal serum which contained a known amount of PhN activity was added to serum of a patient with chronic lymphocytic leukemia.

Four patients with chronic lymphocytic leukemia had recurrent infections caused by pneumococci, *E. coli* microorganisms or herpes zoster virus. No increase in the PhN titers was noted in this group of patients during recovery from an acute infection. Titers of one patient, who had myelogenous leukemia and later developed bacteremia caused by *Staphylococcus aureus* and *E. coli*,

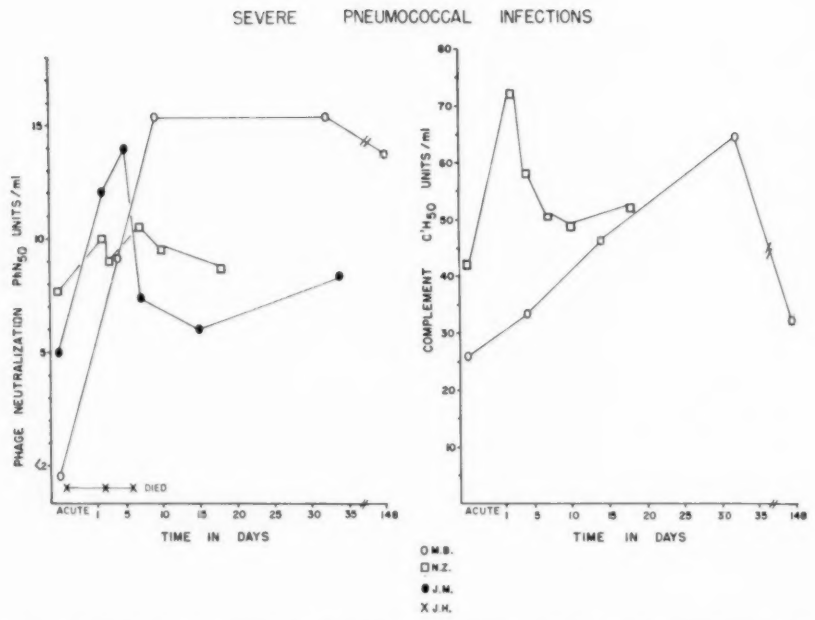

Fig. 2.—Variety of patterns of phage neutralizing activity and complement observed in serial blood samples obtained from patients during acute and convalescent phases of severe pneumococcal infections.

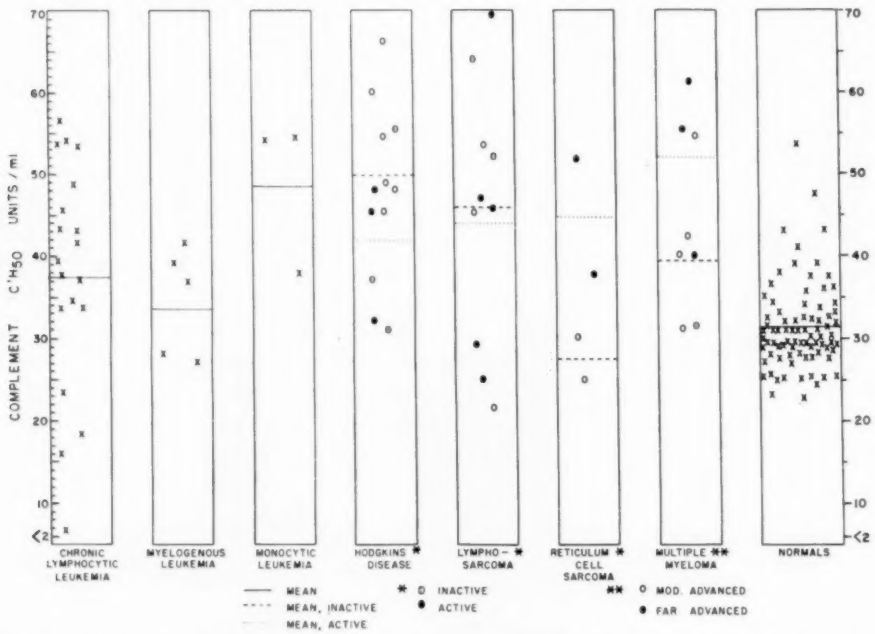

Fig. 3.—Individual and mean values of phage neutralizing activity of normal subjects and patients with leukemia, lymphoma, sarcoma, and multiple myeloma.

decreased from 8.7 to less than 2 PhN_{50} per milliliter during infection. This patient died. Serial PhN levels of one patient with monocytic leukemia, studied at the time of a severe skin infection caused by *Staph. aureus,* showed persistently high levels ranging from 18.1 to 22.2 PhN_{50} per milliliter during a period of 4 weeks.

Serum complement was measured in 20 patients with chronic lymphocytic, 5 patients with myelogenous and 3 patients with monocytic leukemia. Titers in all patients (excluding 3 with chronic lymphocytic leukemia) were normal or high. No anticomplementary activity was found in the sera of patients with chronic lymphocytic leukemia who had low complement titers.

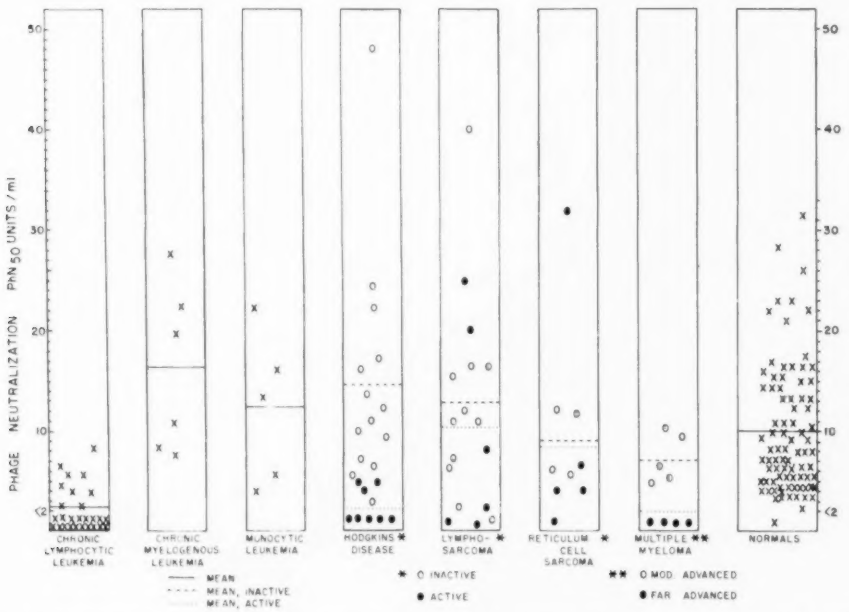

Fig. 4.—Individual and mean values of complement in serum of normal subjects and patients with leukemia, lymphoma, sarcoma, and multiple myeloma.

Complement titers of two patients with chronic lymphocytic leukemia were measured during acute pneumococcal infection. In one patient a titer of less than 2 $C'H_{50}$ per milliliter was obtained. No anticomplementary activity was found. With clinical recovery the complement of this patient did not increase, whereas that of the second patient rose from 23.2 to 37.1 $C'H_{50}$ per milliliter in 7 days.

2. *Lymphomas and sarcomas:* The total number of 47 patients was divided into 2 groups: (a) all patients in the active phase of disease had fever, anemia, adenopathy, and hepatomegaly with or without splenomegaly; (b) all patients in the inactive phase of disease had no fever or anemia, minimal or no adenopathy, and had the diagnosis made by biopsy alone. Serum PhN was measured in 22 patients with Hodgkin's disease (8 with active disease), 16 patients with

lymphosarcoma (6 active), and 9 patients with reticulum cell sarcoma (5 active). All patients with active Hodgkin's disease had PhN titers less than 5 PhN_{50} per milliliter. Four of these 8 acutely ill patients died one to 4 weeks after their sera were studied. Serum PhN of one patient dropped from 7.4 to less than 2 PhN_{50} per milliliter with a change from the inactive to active phase of the disease from which the patient died. In contrast to the invariably low levels of PhN seen during active Hodgkin's disease, titers in patients with sarcoma could not be correlated with the activity of the disease. Titers greater than 5 PhN_{50} per milliliter were present in 5 of 11 patients who had active disease.

Serum complement was measured in 12 patients with Hodgkin's disease (3 with active disease), 9 patients with lymphosarcoma (5 active), and 4 patients with reticulum cell sarcoma (2 active). Titers in all patients were normal or more frequently high regardless of the activity of the disease.

D. *Multiple Myeloma.*—Serum PhN levels were measured in 9 patients. Four patients were acutely ill with severe bone involvement, pathologic fractures, myeloma cells in the peripheral blood, fever, high globulins, and repeated infections. They were considered to be in the far advanced stage of the disease. Two of these patients died. In all 4 patients titers were less than 2 PhN_{50} per milliliter. Serum PhN of one of these patients who had repeated pneumococcal infections failed to increase during the recovery phase of the acute infection. Five other patients with moderately advanced multiple myeloma had minimal bone involvement, myeloma cells in the marrow, high globulins, and no history of recent infections. PhN titers in all of these patients were greater than 5 PhN_{50} per milliliter.

Serum complement was measured in 8 patients with multiple myeloma. The titers were normal or more often high regardless of the stage of the disease.

E. *Paroxysmal Nocturnal Hemoglobinuria (PNH).*—In 2 patients with PNH, studied during remission of the disease, serum PhN titers were 7 and 7.1 PhN_{50} per milliliter and serum complement titers were 31.2 and 34.8 $C'H_{50}$ per milliliter respectively.

DISCUSSION

Until 1957 the measurement of properdin in human serum was based on the ability of properdin to combine with polysaccharides such as zymosan.[7] The technique used in our studies utilizes another property ascribed to serum properdin, i.e., its ability to neutralize viruses. As described by Van Vunakis and collaborators,[2-4] serum which contains properdin can neutralize the activity of T_2r+ bacteriophage active on *E. coli* B. Barlow and co-workers suggested that lack of complete quantitative agreement between the properdin activity obtained by the zymosan and phage assays may be due to serum factors other than complement, properdin, and Mg^{++} needed in the two assays, or, possibly, to differences in sensitivity of the two techniques. The possibility that properdin

is not a single substance but a group of proteins has recently been raised by Cowan.[8] Unfortunately, no report on clinical study correlating the results of the zymosan and phage assays has been published to date.

Our data on serum PhN activity correspond with the studies of Hinz, who, using the zymosan assay for properdin determinations, found low levels of properdin in patients with pneumococcal infections and normal levels in patients with subacute bacterial endocarditis.[9] Our data of serum complement changes in infections confirm the results of Ecker and associates,[10] and Rutstein and Walker,[11] although the techniques used in their studies are markedly different from ours and a quantitative comparison is not possible.

The serum PhN activity studied in our laboratories corresponds with the work of Rottino and Levy,[12, 13] who, using the zymosan assay for properdin determinations, found low levels in "terminal Hodgkin's cases" and in some cases of "lymphoma." Our results are different from their observations made on sera of patients with chronic lymphocytic leukemia. Using the zymosan assay, they found normal levels of properdin in this disease; the vast majority of sera studied in our laboratories by means of the bacteriophage technique revealed low levels of PhN activity. Our observations on serum complement levels in lymphomas correspond with those of Rottino and Levy.[14]

It has been described that properdin, which may reflect phage neutralizing activity of serum, can react with highly branched polysaccharides such as zymosan, pneumococcal capsular polysaccharide, endotoxins, levans, dextrans, and mucin.[15] Because of this property, properdin, in association with other serum factors, may be bactericidal,[16] neutralize viruses,[2-4, 17-19] and lyse abnormal red blood cells such as in paroxysmal nocturnal hemoglobinuria.[20-22] In experimental animals, after the administration of polysaccharides[23-25] or *E. coli* microorganisms,[26, 27] a marked drop in properdin occurs within 2 hours, followed by a marked rise 24 to 48 hours later. Increased resistance to infection is manifested by these experimental animals as long as the properdin levels remain high.[28, 29] In contrast, increased susceptibility to infection and low properdin levels are found after total body irradiation[30] and in hemorrhagic shock.[31, 32]

It is well known that complement may participate in the immunochemical phenomena which occur during antigen-antibody reactions.[33] Complement may enhance phagocytosis,[34] influence agglutination,[35] and, at least in part, be responsible for the bactericidal and hemolytic activity of serum.[36, 37]

Thus, it would appear that, at least early in infection, substances such as properdin and complement may be utilized in host resistance to infection. The mechanism and significance of the change in titer of these substances is not clear. Diminished levels of properdin may be due to its reaction with polysaccharides; diminished levels of complement may be due to its fixation in antigen-antibody reactions. Increased levels of properdin and complement with recovery from acute infections may reflect overproduction or decreased utilization of these substances. Persistently low or declining titers are seen frequently in individuals who die from their infection.

Low serum PhN activity titers observed in patients with certain malignant diseases may explain in part the increased susceptibility to infections manifested by patients with chronic lymphocytic leukemia, active Hodgkin's disease, and far advanced multiple myeloma. The lack of increase in PhN activity titer in these patients during recovery from an acute infection may indicate their inability to produce or utilize this substance. Elevated complement titers in most malignant disorders studied cannot be explained satisfactorily with our present understanding of complement.

CONCLUSIONS AND SUMMARY

Bacteriophage neutralizing activity of serum (PhN), in most cases:

1. Is low in severe pneumococcal infections and bacteremia caused by *Staphylococcus aureus* or gram-negative microorganisms. A two- to ninefold increase in titer within 48 hours with a continuous rise for as long as 4 weeks is seen in patients who recover.

2. Is normal or high in patients studied during recovery from an acute pneumococcal infection or bacteremia caused by *Staph. aureus* or gram-negative microorganisms, as well as in patients with subacute bacterial endocarditis, lung abscess, and empyema.

3. Is low in chronic lymphocytic leukemia, active Hodgkin's disease, far advanced multiple myeloma, and some cases of active reticulum cell and lymphosarcoma.

4. Is normal or high in chronic myelogenous, monocytic leukemia, moderately advanced multiple myeloma, and most cases of reticulum cell and lymphosarcoma.

Titers of serum complement, using $C'H_{50}$ unit system, in most cases are:

1. Normal or decreased during acute infections. A two- to threefold increase in titer within 48 hours with a continuous rise for as long as 4 weeks is seen in patients who recover.

2. Normal or high in chronic lymphocytic (excluding 3 cases in this series), myelogenous and monocytic leukemia, Hodgkin's disease, reticulum cell sarcoma, lymphosarcoma, and multiple myeloma regardless of the activity of the disease.

It is suggested that phage neutralizing activity (PhN) of serum may reflect properdin. The possible mechanisms and significance of change in properdin and complement titers in infections and malignant disorders are discussed.

We wish to express our appreciation to Dr. Alvin Marucci for his valuable suggestions and assistance in the serum complement study, and to Drs. Eugene Lozner and Frank Furth for their interest and cooperation in the study of patients with blood dyscrasias and lymphomas.

REFERENCES

1. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C.: Properdin System and Immunity. I. Demonstration and Isolation of a New Serum Protein, Properdin, and Its Role in Immunologic Phenomena, Science **120**: 270, 1954.
2. Van Vunakis, H., Barlow, J. L., and Levine, L.: Neutralization of Bacteriophage by the Properdin System, Proc. Nat. Acad. Sc. **42**: 391, 1956.

3. Barlow, J. L., Van Vunakis, H., and Levine, L.: Studies on the Inactivation of Phage by the Properdin System. I. Evidence for Complement, Properdin and Magnesium Requirements, J. Immunol. **80**: 339, 1957.
4. Barlow, J. L., Van Vunakis, H., and Levine, L.: Studies on the Inactivation of Phage by the Properdin System. II. Quantitative Assay of Phage Neutralizing Activity, J. Immunol. **80**: 349, 1957.
5. Osler, A. G., Strauss, J. H., and Mayer, M. M.: Diagnostic Complement Fixation. I. A Method, Am. J. Syph., Gonor. & Ven. Dis. **36**: 140, 1952.
6. Rapp, H. J.: Purification and Immunochemical Characterization of the Heat Stable, Alcohol-Soluble Hemolytic Antibody Inhibitor of the Sheep Erythrocyte, Thesis prepared at Johns Hopkins School of Hygiene and Public Health, 1953.
7. Pillemer, L., Blum, L., Lepow, I. H., and Wurz, L.: The Properdin System and Immunity. III. The Zymosan Assay of Properdin, J. Exper. Med. **103**: 1, 1956.
8. Cowan, K. M.: Interpretation of Properdin Levels Determined by Phage Neutralization Technique, Science **128**: 778, 1958.
9. Hinz, C. F.: Properdin Levels in Infectious and Non-infectious Disease, Ann. New York Acad. Sc. **66**: 268, 1956.
10. Ecker, E. E., Seifter, S., Dozois, T. F., and Barr, L.: Complement in Infectious Disease in Man, J. Clin. Invest. **25**: 800, 1946.
11. Rutstein, D. D., and Walker, W. H.: Complement Activity in Pneumonia, J. Clin. Invest. **21**: 347, 1942.
12. Rottino, A., and Levy, A. L.: Serum Properdin Levels in Patients With Hodgkin's Disease and Carcinoma, Cancer **10**: 877, 1957.
13. Rottino, A., Levy, A. L., and Conte, A.: The Study of Serum Properdin Levels of Patients With Malignant Tumors, Cancer **11**: 351, 1958.
14. Rottino, A., and Levy, A. L.: Behavior of Total Serum Complement in Hodgkin's Disease and Other Malignant Lymphomas, Blood **14**: 246, 1959.
15. Pillemer, L., Schoenberg, M., Blum, L., and Wurz, L.: Properdin System and Immunity. II. Interaction of the Properdin System With Polysaccharides, Science **122**: 545, 1955.
16. Wardlaw, A. C., and Pillemer, L.: The Properdin System and Immunity. V. The Bactericidal Activity of the Properdin System, J. Exper. Med. **103**: 553, 1956.
17. Wedgwood, R. J., Ginsberg, H. S., and Pillemer, L.: Properdin System and Immunity. VI. Inactivation of Newcastle Disease Virus by Properdin System, J. Exper. Med. **104**: 707, 1956.
18. Wedgwood, R. J., Ginsberg, H. S., Seibert, R. H., and Pillemer, L.: The Inactivation of the Newcastle Disease Virus by the Properdin System, Am. J. Dis. Child. **90**: 508, 1955. (Abst.)
19. Ginsberg, H. S., and Wedgwood, R. J.: Inactivation of the Virus by the Properdin System, Ann. New York Acad. Sc. **66**: 251, 1956.
20. Hinz, C. F., Jordan, W. S., and Pillemer, L.: The Properdin System and Immunity. IV. The Hemolysis of Erythrocytes From Patients With Paroxysmal Nocturnal Hemoglobinuria, J. Clin. Invest. **35**: 453, 1956.
21. Hinz, C. F., and Pillemer, L.: The Requirement for the Properdin System in the Hemolysis of Human Erythrocytes Treated With Tannic Acid, J. Clin. Invest. **34**: 912, 1955.
22. Blaisdell, R. K., Priest, R. E., and Beutler, E.: Paroxysmal Nocturnal Hemoglobinuria: A Case Report With a Negative Ham Presumptive Test Associated With Serum Properdin Deficiency, Blood **13**: 1074, 1958.
23. Landy, M., and Pillemer, L.: Elevation of Properdin Levels in Mice Following Administration of Bacterial Lipopolysaccharides, J. Exper. Med. **103**: 823, 1956.
24. Landy, M., and Shear, M. J.: Similarity of Host Responses Elicited by Polysaccharides of Animal and Plant Origin and by Bacterial Endotoxins, J. Exper. Med. **106**: 77, 1957.
25. Pillemer, L., Landy, M., and Shear, M. J.: The Properdin System and Immunity. VII. Alterations in Properdin Levels and Resistance to Infections in Mice Following the Administration of Tissue Polysaccharides, J. Exper. Med. **106**: 99, 1957.
26. Rowley, D.: Stimulation of Natural Immunity to E. coli Infections, Observations on Mice, Lancet **1**: 232, 1955.
27. Rowley, D.: Rapidly Induced Changes in the Levels of Non-specific Immunity in Laboratory Animals, Brit. J. Exper. Path. **37**: 223, 1956.
28. Landy, M., and Pillemer, L.: Increased Resistance to Infection and Accompanying Alteration in Properdin Levels Following Administration of Bacterial Lipopolysaccharides, J. Exper. Med. **104**: 383, 1956.
29. Landy, M.: Increase in Resistance Following Administration of Bacterial Lipopolysaccharides, Ann. New York Acad. Sc. **66**: 292, 1956.
30. Ross, O. A.: The Properdin System in Relation to Fatal Bacteremia Following Total Body Irradiation of Laboratory Animals, Ann. New York Acad. Sc. **66**: 274, 1956.

31. Frank, E., Fine, J., and Pillemer, L.: Serum Properdin Levels in Hemorrhagic Shock, Proc. Soc. Exper. Biol. & Med. **89**: 223, 1955.
32. Fine, J.: Effect of Peripheral Vascular Collapse on the Antibacterial Defense Mechanisms, Ann. New York Acad. Sc. **66**: 329, 1956.
33. Kabat, E. A., and Mayer, M. M.: Immunochemistry, Springfield, Ill., 1948, Charles C Thomas, Publisher, pp. 127-133.
34. Nelson, R. H., and Lebrun, J.: The Requirement for Antibody and Complement for in Vitro Phagocytosis of Starch Granules, J. Hyg. **54**: 8, 1956.
35. Thjötta, H., and Jonsen, J.: The Influence of Complement Upon Agglutination, Acta path. et microbiol. scandinav. **26**: 326, 1949.
36. Osborn, T. W. B.: Complement or Alexin, London, 1939, Oxford University Press, pp. 25-36.
37. Ecker, E. E., Seifter, S., and Dozois, T. F.: Human Complement, J. LAB. & CLIN. MED. **30**: 39, 1945.

CYCLOSERINE: GROWTH ENHANCEMENT PHENOMENON FOUND DURING SENSITIVITY STUDIES: CLINICAL IMPLICATIONS

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In the search for new drugs to treat tuberculosis, cycloserine, a strain of Streptomyces was discovered. This drug because of its toxicity in man has been used only when the established agents were not effective.

In this study 45 of 62 patients whose sputum cultures were subjected to cycloserine sensitivity reactions were found to be initially resistant to 20 mcg. per milliliter. None of the patients had received the antibiotic.

In the Lowenstein-Jensen test medium that contained 5, 10, or 20 mcg. per milliliter of cycloserine it was observed, in most cases, that the growth of tubercle bacilli was equal to or noticeably more luxuriant than the controls. This would indicate an enhancement of growth in the low concentrations of cycloserine.

Although in vitro studies do not always indicate similar in vivo results, this work suggested that in the use of cycloserine in the treatment of tuberculosis, large doses should be used to bring the blood level to at least 30 mcg. per milliliter. It should also be accompanied with a secondary antibiotic to which the patient is sensitive to obtain the cumulative effect of both drugs.

NUMEROUS studies have been made of the antibiotic effectiveness of cycloserine against infections involving *Mycobacterium tuberculosis*. Dunner[1] comparing cycloserine and isoniazid (INH) with INH and para-aminosalicylic acid (PAS), concluded that the cycloserine combination gave consistently lower incidence of cavity closure, particularly among those with large cavities, considerably reduced occurrence of reversal of infection in those patients with medium or large cavities, and considerably higher frequency of advancing areas of infection as revealed by roentgenographic examination. From studies with experimental animals Wolinsky and Steenken[2] concluded that cycloserine alone was completely ineffective in the guinea pig. Furthermore, it was stated that the treated animals died more rapidly than the controls without antibiotic. Patnode and associates,[3] also working with guinea pigs, reported that the tubercle bacilli were recovered in equal numbers from the spleens of cycloserine treated and untreated animals.

The present report describes a series of susceptibility studies made to test, in vitro, the bacteriostatic action of cycloserine, as compared with INH,

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streptomycin, and PAS antibiotics, on virulent strains of *Myco. tuberculosis.* Concomitant with the sensitivity studies, experiments were performed to determine the stability of cycloserine after undergoing the manipulatons necessary for its incorporation into Lowenstein-Jensen tuberculosis medium. Cultures prepared in the course of stability testing were used to augment the data on sensitivity reactions. The possibility of degenerative changes caused by heat, storage, and selected diluents, which resulted in loss of antibiotic potency was explored. It is believed the facts obtained correlate closely with the work of the individuals cited previously, and that this additional information strongly suggests an explanation of the clinical and experimental phenomena which they observed. Other implications regarding the clinical use of cycloserine have been derived from this work.

MATERIALS AND METHODS

Throughout the study, three samples of cycloserine were used. Two samples, called Seromycin,* were received, one in 1956, hereafter called S-56, and another in 1958, hereafter called S-58. Oxamycin,† received in 1957, will be designated O-57.

1. *Susceptibility Tests.*—Organisms used were from 62 patients diagnosed with virulent strains of *Myco. tuberculosis.* These patients were not on cycloserine chemotherapy, but were treated according to protocol with INH, streptomycin, and PAS. A total of 120 cultures from these 62 patients were tested.

The sensitivity procedures used were those described in the Tuberculosis Laboratory Methods of the Fifteenth Conference on Chemotherapy. Lowenstein-Jensen medium was used. The indirect testing technique was employed, and readings were made weekly. Final results were determined when the control tubes without antibiotic supported at least a 3 plus growth. Growth on control was then compared with growth on media that contained cycloserine in concentration of 5, 10, and 20 mcg.

2. *Stability Tests.*—

a. *Effect of heat sterilization on cycloserine:* This test was performed to establish that the growth on Lowenstein-Jensen medium that contained cycloserine was not attributable to destruction of the antibiotic properties during heat sterilization (inspissation). Kirchner liquid medium‡ was used. Some of the liquid media that contained antibiotic was sterilized by filtration through a bacterial ultrasintered glass filter and some by autoclaving at 10 pounds for 10 minutes.

b. *Determinations of tubercular growth on stored cycloserine media:* In an effort to determine the effect of the age of the cycloserine stock solution and cycloserine working media on growth of tubercle bacilli, Lowenstein-Jensen medium with concentrations of 5, 10, and 20 mcg. per milliliter was made from stock solutions which had been stored. Some of the media were inoculated immediately and some were stored and inoculated later. Growth in all cases was checked against control tubes without antibiotics. It is significant to report that with these cultures, as with those produced for clinical sensitivity study, growth in the media that contained cycloserine, when growth appeared, was often noticeably more profuse than that of control, or was at least equal. This is evidenced by the photographs of Cases I and II where growth was more profuse in the cycloserine media.

RESULTS

In Table I, which shows the results of susceptibility tests, resistance to

*Eli Lilly & Company.

†Merck & Company, Inc.

‡Formula given by Mackie, T. J., and McCartney, J. E.: in *Handbook of Practical Bacteriology*, ed. 9, Edinburg, 1953, E. & S. Livingstone, Ltd., p. 198.

TABLE I. SUSCEPTIBILITY OF MYCO. TUBERCULOSIS TO CYCLOSERINE, AS COMPARED TO INH, STREPTOMYCIN, AND PAS IN RETREATMENT CASES

INITIALLY RESISTANT TO CYCLOSERINE (5, 10, AND 20 MCG./ML.)	PATIENTS (45)	CULTURES (85)	SPECIFIC CULTURE SENSITIVITY REACTIONS	
INH, Streptomycin or *PAS.*—				
A. Sensitive to 1; resistant to 2 antibiotics	6	17	Sen. to INH; Res. to S. & PAS	3
			Sen. to S.; Res. to INH & PAS	6
			Sen. to PAS; Res. to INH & S.	8
B. Sensitive to 2; resistant to 1 antibiotic	15	32	Sen to S. & PAS; Res. to INH	21
			Sen. to INH & PAS; Res. to S.	6
			Sen. to INH & S.; Res. to PAS	5
C. Sensitive to 3 antibiotics	21	28		
D. Resistant to 3 antibiotics	3	8		
Sensitive to 20 mcg./ml. of cycloserine	(17)	(35)		
INH, Streptomycin or *PAS.*—			*Sen. to INH; Res. to S. & PAS	2
A. Sensitive to 1; resistant to 2 antibiotics	2	11	Sen. to S.; Res. to INH & PAS	7
			Sen. to PAS; Res. to INH & S.	2
B. Sensitive to 2; resistant to 1 antibiotic	5	8	Sen. to S. & PAS; Res. to INH	8
			Sen. to INH & PAS; Res. to S.	0
			Sen. to INH & S.; Res. to PAS	0
C. Sensitive to 3 antibiotics	10	16		
D. Resistant to 3 antibiotics	0	0		
Total examinations	(62)	(120)		

*Sen. = sensitive; Res. = resistant. INH, 0.2, 1, 5 mcg. per milliliter. S. = streptomycin, 3.5, 10, 100 mcg. per milliliter. PAS, 5, 10, 100 mcg. per milliliter.

cycloserine occurs early in patients treated with the three antituberculates. In 45 of the 62 patients, with 85 cultures, there was initial resistance to cycloserine, whereas 17 patients with 35 cultures were susceptible to a minimum of 20 mcg. per milliliter and resistant to the two lower concentrations. All of the 17 initially sensitive to cycloserine were also sensitive to at least one of the other three antibiotics. Three of the 62 patients yielded organisms resistant to all four of the drugs used.

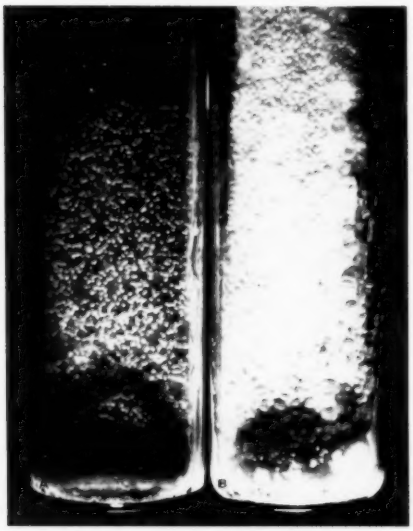

Fig. 1.—Case I. *A*, Control (no antibiotic). *B*, Cycloserine, 10 mcg. per milliliter.

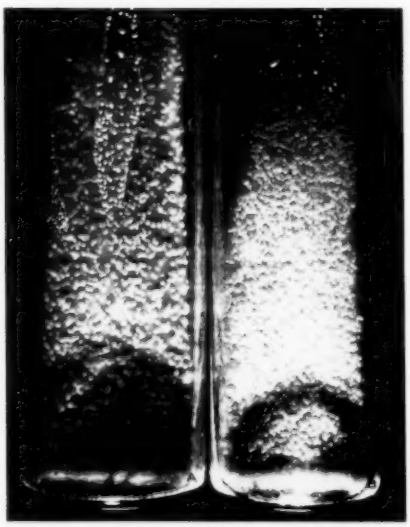

Fig. 2.—Case II. *A*, Control (no antibiotic). *B*, Cycloserine, 20 mcg. per milliliter.

Further breakdown of Table I shows the specific drugs to which the cultures reacted. Of the 85 cultures with a resistant reaction to 20 mcg. per milliliter of cycloserine, 43 cultures were also resistant to INH, 25 were resistant to streptomycin, and 22 were resistant to PAS. These cultures were from patients who had been on treatment protocol to the three common antibiotics for some time and had set up their resistance independent of cycloserine.

It was noted that those 17 patients who showed initial sensitivity to cycloserine were sensitive to a minimum concentration of 20 meg. per milliliter. In the tubes that contained the two lower concentrations of the drug, 5 and 10 meg. per milliliter, growth was either equal to or significantly more abundant than that in the control tubes. Organisms obtained from 45 of the patients were initially resistant to cycloserine, up to 20 meg. per milliliter. In the case of these organisms, media that contained any of the three concentrations, 5, 10, or 20 meg. per milliliter, supported in almost every case growth either equal to or noticeably more luxuriant than on control.

The striking appearance of growth enhancement, as demonstrated in Cases I and II, was very easily detected when the sensitivity reactions in a complete antibiotic series were compared (Figs. 1 and 2). The growth was so luxuriant that the tubes that contained the cycloserine could be picked from the series without identification.

When the stability of cycloserine was studied it was found that the antibiotic sterilized by heat or filtration made no difference in the growth pattern of the tubercle bacilli. Neither was there a difference in growth pattern on fresh antibiotic media compared with stored media in the lower concentrations of the drug.

DISCUSSION

It would appear desirable, after a review of the literature and in light of the aforementioned results, to refrain from prescribing clinical dosages of cycloserine for patients with tuberculosis prior to making thorough sensitivity studies of the organism strains yielded. These sensitivity studies should include INH, streptomycin, and PAS, as well as cycloserine. Such workers as Welch,[4] Barclay and Russe,[5] Epstein,[6] Lester,[7] Toguri,[8] and Wolinsky[9] feel that the best results with cycloserine are derived when the antibiotic is combined with one of the more common drugs to which the patient is sensitive.

In a group of 62 patients previously treated with INH, streptomycin, and PAS, and resistant to at least one of these, there is a high incidence (72 per cent) of initial resistance to 20 meg. per milliliter of cycloserine. These patients have never received cycloserine. It is possible that resistance to 20 meg. per milliliter in vitro does not indicate resistance in vivo. The opinion today is that resistance to cycloserine occurs somewhere above 30 meg. per milliliter. Therefore, larger doses of cycloserine should be used to bring blood serum concentrations above the 30 meg. per milliliter level.

Pyle and associates[10] by using high dosage of cycloserine (30 mg. per kilogram), that is 1 to 2.5 Gm. daily, in salvage cases were able to improve over 50 per cent of their patients so that they could be operated upon. In spite of the use of anticonvulsive drugs, convulsions were high in the series of 50 patients.

These in vitro observations are in accord with the clinical and experimental findings outlined by Dunner,[1] Wolinsky and Steenken,[2] and Patnode and associates.[3]

SUMMARY

The evidence produced in this study indicates that sensitivity occurs within some optimum concentration range for cycloserine, but a lower concentration of the drug may have a significant enhancing effect. This is evidenced by those organisms which showed initial resistance to cycloserine where the patients' strain exhibited abundant growth on the 5 and 10 mcg. per milliliter of medium that contained cycloserine. Growth, when it appeared, was either equal to or significantly more abundant than in the control tubes without antibiotic.

When growth of tubercle bacilli was found, fresh working media that contained 5, 10, and 20 mcg. per milliliter of cycloserine supported equal growth as that of stored media. Heat sterilization did not seem to affect the test media.

The best diluent for cycloserine was found to be distilled water, saline, or Dubos broth (buffered). As stated by Steenken and Wolinsky,[11] alkaline suspensions of cycloserine were found to be more stable than acid and neutral solutions.

REFERENCES

1. Dunner, E.: Highlights of the Seventeenth VA-Armed Forces Conference on the Chemotherapy of Tuberculosis, Antibiotic Med. **5**: 270, 1958.
2. Wolinsky, E., and Steenken, W., Jr.: Cycloserine Alone and in Combination With Other Drugs in Experimental Guinea Pig Tuberculosis, Am. Rev. Tuberc. **75**: 510, 1957.
3. Patnode, R. A., Hudgins, P. C., and Cummings, M. M.: Effect of Cycloserine on Experimental Tuberculosis in Guinea Pigs, Am. Rev. Tuberc. **72**: 117, 1955.
4. Welch, H., Putnam, L. E., and Randall, W. A.: Antibacterial Activity and Blood and Urine Concentrations of Cycloserine Following Oral Administration, Reported at the Fourteenth VA-Army-Navy Conference on the Chemotherapy of Tuberculosis, February, 1955, p. 314.
5. Barclay, W. R., and Russe, H.: The in Vitro Action of Cycloserine on M. tuberculosis, Am. Rev. Tuberc. **72**: 236, 1955.
6. Epstein, I. G., Nair, K. G. S., and Boyd, L. J.: Cycloserine, A New Antibiotic, in the Treatment of Human Pulmonary Tuberculosis: A Preliminary Report, Antibiotic Med. **1**: 80, 1955.
7. Lester, W., Jr., Salomon, A., Reimann, A. F., Shulruff, E., and Berg, G. S.: Cycloserine Therapy in Tuberculosis in Humans, Am. Rev. Tuberc. **74**: 121, 1956.
8. Toguri, E., and Atwell, R. J.: Cycloserine Combined With Pyrazinamide in the Treatment of Pulmonary Tuberculosis, Am. Rev. Tuberc. **78**: 927, 1958.
9. Wolinsky, E., and Kapur, V. N.: Evaluation of Cycloserine With High Dosage of Isoniazid in Chronic Treatment—Failure Pulmonary Tuberculosis, Am. Rev. Tuberc. **80**: 269, 1959.
10. Pyle, M. M., Pfuetze, K. H., Barclay, W. R., and Kasik, J. E.: Cycloserine in High Dosage in "Salvage Cases" of Pulmonary Tuberculosis With Control of Toxicity by Concomitant Medication: Efficacy as Medical and Pre-surgical Treatment, Reported at the Eighteenth VA-Armed Forces Conference on the Chemotherapy of Tuberculosis, February, 1959, pp. 128-137.
11. Steenken, W., Jr., and Wolinsky, E.: Cycloserine: Antituberculous Activity in Vitro and in the Experimental Animal, Am. Rev. Tuberc. **73**: 539, 1956.

AN ASSAY OF ANTIHEMOPHILIC GLOBULIN ACTIVITY IN THE CARRIER FEMALE

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By using a modification of Quick's erythrocytin assay for antihemophilic globulin, twenty-one definite carriers and five probable carriers were assayed for plasma antihemophilic globulin levels. It was found that twenty-three of the subjects showed abnormally low antihemophilic globulin levels (30 to 40 per cent) as compared to normal subjects (50 to 200 per cent of the mean). These low levels were further characterized by the clinical manifestations of these carriers. Seventy-seven per cent of the carriers had a subjective history of easy bruising, 11.5 per cent had prolonged bleeding after surgical operation, and 7 per cent had prolonged bleeding after tooth extractions.

In family studies, it was found that the antihemophilic globulin activity developed by carriers in the same family and even throughout several generations showed similar plasma antihemophilic globulin levels which suggested that the exact plasma antihemophilic globulin level is evidently a genetically controlled factor.

SINCE Hay,[1] in 1813 described the first pedigree of hemophilia in the Appleton-Swain family, many attempts have been made to demonstrate an abnormal coagulation mechanism in the human carrier female. These have ranged from clotting times[2, 3] to the more refined studies of the thromboplastin generation test,[4-7] prothrombin consumption,[8] plasma recalcification times,[6] and antihemophilic globulin[9] (AHG) assays. Some of these studies have shown a slight coagulation defect but most reports have failed to confirm conclusively that carriers have coagulation mechanisms different from the normal mechanism. However, Pitney and Arnold[7] recently used a sensitive modification of the thromboplastin generation test and showed that a mild deficiency of AHG existed in a majority of female carriers. Therefore, if one is to demonstrate this possible AHG deficiency in carriers, it seems imperative to have a very sensitive assay method for AHG.

Shinowara,[10] Quick and co-workers,[11] and Georgatsos and associates[12] have shown that there is a clotting factor in human red blood cell stroma, termed "erythrocytin" by Quick,[13] which is liberated after hemolysis of erythrocytes.

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This factor has been shown to have a very weak thromboplastic action as it will change only 7 per cent of 1 unit of prothrombin to thrombin when substituted for thromboplastin in the normal one stage prothrombin time.[10] However, there is presumably an interaction of erythrocytin with antihemophilic globulin (AHG) in platelet poor normal plasma which causes a marked increase in the prothrombin consumption values as measured by the method of Quick.[13] In contradistinction, if this erythrocytin is added to platelet poor plasma from a patient with AHG deficiency, there is no change in the prothrombin consumption values. Therefore, if increasing minute amounts of normal plasma are added to a substrate of erythrocytin and hemophiliac plasma, there is a direct linear relationship of increasing prothrombin consumption values to the increasing amounts of normal plasma added. By this technique, the minute difference between the AHG content of 0.0005 and 0.0008 ml. of normal plasma can be detected.

The purpose of this study is to determine whether, by applying this method, a deficiency in the plasma AHG content in carrier females can be detected.

CLINICAL MATERIAL

The hemophiliac patients were previously proved to be AHG deficient by the thromboplastin generation test. Unless otherwise specified, all had severe hemophilia and did not have a partial deficiency of AHG. None of the patients could be classified as having "vascular hemophilia" as they had normal bleeding times.

The criteria established for the definition of carriers were similar to those of Merskey and MacFarlane.[8] Definite carriers were established as: (1) mothers who had two or more children with either hemophilia and/or proved carrier states; (2) mothers who only had either a hemophiliac or a proved carrier offspring but who also had a positive family history of classic hemophilia; (3) daughters of hemophiliac men. The probable carriers were defined as mothers who had one hemophiliac or proved carrier offspring but no other family history.

MATERIAL AND METHODS

The *substrate* for the test consisted of erythrocytin, hemophilic plasma, and aged serum.

The *hemolyzate* which contained *erythrocytin* was prepared by the method of Georgatsos.[12] Nine volumes of blood were collected in a siliconized syringe and mixed with 1 volume of 0.1M sodium oxalate solution. This was centrifuged for 5 minutes at 1,000 r.p.m., and the plasma and buffy coat removed. The plasma was replaced with an equal volume of 0.85 per cent saline and mixed thoroughly by tilting gently. The mixture was again centrifuged at 1,000 r.p.m. for 5 minutes and the supernatant was removed. The cells were resuspended by tilting in an equal volume of 0.85 per cent saline, and the mixture was centrifuged at 2,000 r.p.m. for 20 minutes. This step was repeated three times. At the end of this time, the cells were resuspended in a volume of saline equal to the original plasma volume, vigorously mixed by stirring, and frozen at -20° C. for 12 hours. The hemolyzate was then thawed and distributed in 1 ml. aliquots and stored at -20° C. It can be repeatedly thawed and refrozen without losing its potency.

AHG-deficient plasma for the substrate was obtained from known hemophiliac male patients who were shown to have a prothrombin consumption time between 8 to 10 seconds as shown by the method of Quick.[13] If the substrate plasma had a prothrombin consumption value above 8 to 10 seconds, the resulting erythrocytin prothrombin consumption values were above the range of normal. The blood was drawn and mixed thoroughly but gently with 1 ml. of 0.1M sodium oxalate solution per 10 ml. of blood. This was centrifuged at 3,500 r.p.m.

for 20 minutes, and the plasma was then immediately frozen at -20° C. in 1 ml. aliquots. Only two donors were used for the source of hemophilic plasma throughout the study. Their plasmas gave identical prothrombin consumption values.

Aged serum was prepared by the method of Quick.[13]

The quantities of *substrate* material consisted of 0.1 ml. of hemolyzate which contained erythrocytin, 1.0 ml. of AHG-deficient plasma, and 0.001 ml. of aged serum.

Test plasma was obtained by drawing 5 ml. of blood from the patient in a fasting condition (normal, hemophiliac, or suspected carrier) by means of a siliconized syringe and needle. This 5 ml. of blood was added to 0.5 ml. of 0.1M sodium oxalate solution and was immediately centrifuged at 10,000 r.p.m. in the International refrigerated centrifuge at 4° C. for 10 minutes. The resultant platelet poor plasma was carefully pipetted off and was either used immediately or was rapidly frozen by immersion in a dry ice and acetone mixture for later use. The plasmas that had been frozen were thawed by placing in a water bath at 37° C.

The dilution of the test plasma was made just prior to beginning the procedure by accurately pipetting 0.11 ml. of test plasma into 9.9 ml. of 0.85 per cent saline. The resulting diluted plasma was added in aliquots of 0.01, 0.025, 0.05, 0.08, and 0.1 ml. to tubes which contained the substrate material.

The test plasma and substrate mixture were allowed to incubate together at 37° C. for 1 minute and then 0.1 ml. of 0.25M calcium chloride solution was added. The tubes were carefully observed for formation of a solid clot. Exactly 15 minutes after clotting occurred, the clot was loosened with a stirring rod. The tube was centrifuged at 2,500 r.p.m. for 1 minute and returned to the water bath and incubated for 45 additional minutes.

The serum in each tube was then measured for prothrombin content by the method of Quick.[13]

The source of fibrinogen was human plasma treated with barium sulfate prepared by mixing 100 mg. of barium sulfate per 1 ml. of plasma, shaking constantly for 5 minutes, centrifuging, and then using the supernatant plasma.

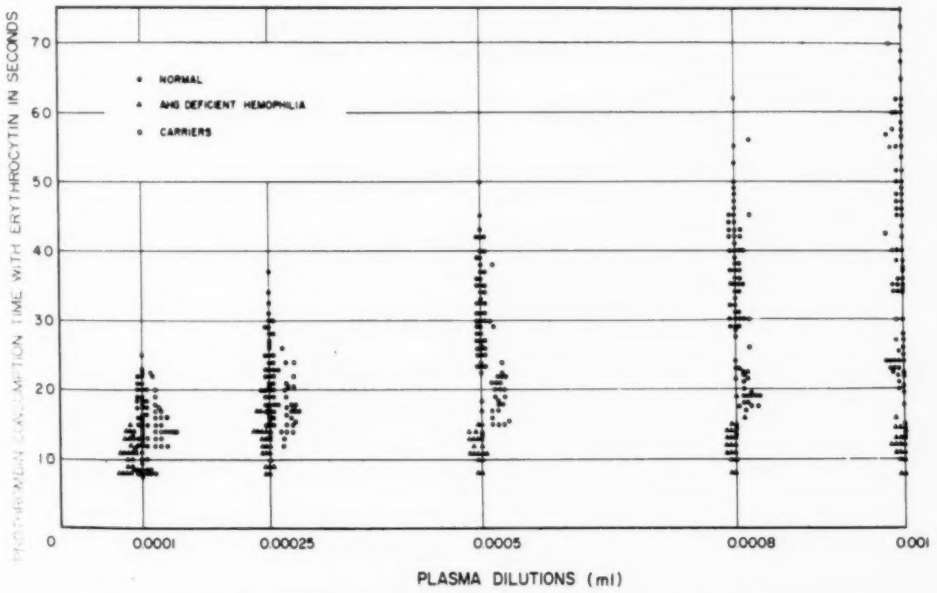

FIG. 1.—Demonstration of the erythrocytin prothrombin consumption times in seconds at various plasma dilutions of normal, female carriers, and AHG hemophiliac patients.

One tenth milliliter of plasma treated with barium sulfate, 0.1 ml. rabbit brain thromboplastin (Difco Laboratories), and 0.1 ml. of 0.025M calcium chloride solution were mixed and allowed to incubate for a few seconds at 37° C. One tenth milliliter of the test serum was blown into the mixture, and the time in seconds until the first formation of fibrin strands was noted. The resultant time was the erythrocytin prothrombin consumption time.

There was no difference from the original AHG activity in test plasma of normal subjects, hemophiliac patients, or carrier individuals after rapid freezing, storage, and later, thawing.

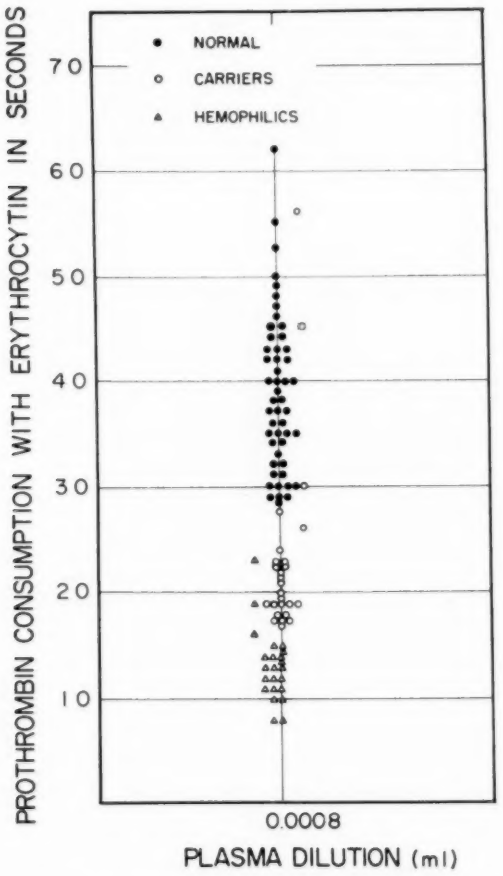

Fig. 2.—Demonstration of the erythrocytin prothrombin consumption times in seconds at 0.0008 ml. dilution of normal, AHG hemophiliac, and female carrier plasmas.

RESULTS

The normal range of erythrocytin prothrombin consumption time was determined on 30 male and 20 female subjects. These results are shown in Fig 1. As increasing amounts of normal plasma were added to the test substrate of erythrocytin, hemophilic plasma, and aged serum, the prothrombin consumption in seconds steadily rose. The average time at 0.0001, 0.0005, 0.0008

and 0.001 ml. was 14.4, 31.6, 38.4, and 48.7 seconds, respectively. This rise was presumed to be due to the increasing amounts of AHG added. There was no difference between the range and average of normal male and female patients.

In addition, the variations of 2[illegible] hemophiliac patients are shown in Fig. 1. In contrast to the results obtained when normal plasma was used, increasing amount of hemophilic plasma (AHG deficient) did not increase the prothrombin consumption. This was presumably due to the absence of AHG in the tested hemophilic plasma.

Thus, it seems evident that one is actually measuring AHG activity in this test system, and the test is sensitive enough to determine the difference in the AHG activity between a dilution of 0.0005 and 0.0008 ml. of normal test plasma.

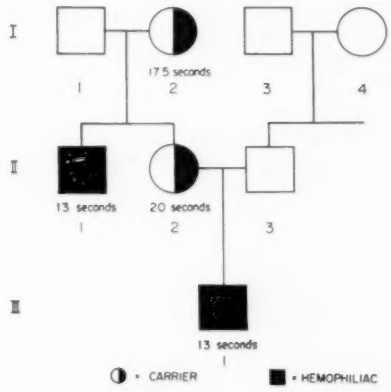

Fig. 3.—Family tree showing heritage and prothrombin consumption at 0.0008 ml. plasma dilution. Lower range of normal, 29 seconds.

Also in Fig. 1, the prothrombin consumption activity produced by plasma of female carriers is recorded. Fig. 2 shows the specific differences obtained when 0.0008 ml. of test plasma was used. All except three of the carriers fall in the area between the normal range and the hemophiliac range. These differences are statistically significant as shown in Table I. At the dilutions of 0.0005, 0.0008, and 0.001 ml., the differences between carrier and normal subjects and the differences between carrier and hemophiliac subjects show a p value of less than 0.0001.

If the mean values of the normal control subjects from Fig. 1 are plotted, using the 0.1 value as 100 per cent, a fairly good linear relationship is obtained. The data thus suggest that the normal range of AHG activity is wide, approximately 50 to 200 per cent of the mean. This is in agreement with the range reported by others.[7] By this method, most of the carrier subjects fall in the 30 to 40 per cent range of AHG activity.

Fig. 3 shows the AHG levels for 3 generations in one family. Notice that the son and the grandson have identical AHG assay levels, and the mother and the daughter have almost identical amounts. This was the only family where

we were able to assay 3 generations in the same family. However, the constancy of AHG levels in families is further shown in Table II. In Family 1, all the sisters have the same prothrombin consumption values. Their affected hemophiliac offspring have erythrocytin prothrombin consumption values of 11.8 and 9 seconds. The remaining two families demonstrate that the erythrocytin prothrombin consumption values of subjects with hemophilia born of the same carriers are identical (Family 2: 10 and 11 seconds, Family 3: 14 seconds).

TABLE I. STATISTICAL EVALUATION OF MEANS AND STANDARD DEVIATIONS OF EACH GROUP

AMOUNT PLASMA FROM PATIENT ADDED TO SUBSTRATE (ML.)	GROUP	PROTHROMBIN CONSUMPTION TIME (SEC.)				
		NORMAL	MEAN	S.D.	CONSTANT	P
0.0005	Control	50	32.28	6.48	9.27	<0.0001
	Carrier	26	19.86	5.		
	Hemophiliac	25	11.9	2.35	7.30	<0.0001
0.0008	Control	50	38.88	7.37	8.6	<0.0001
	Carrier	26	22.97	7.8		
	Hemophiliac	25	12.12	2.65	6.7	<0.0001
0.001	Control	50	49.12	11.13	7.8	<0.0001
	Carrier	26	27.63	11.5		
	Hemophiliac	25	12.24	2.65	6.7	<0.0001

TABLE II

FAMILY	RELATION	AHG STATUS	CASE NO. IN TABLE I	PROTHROMBIN CONSUMPTION AT 0.0008 ML. IN (SEC.)
1	Sisters	Carrier	1	18
			8	18
			9	17
	Cousins	Hemophiliac	1	11
			8 (a)	8
			8 (b)	9
2	Brothers	Hemophiliac	6 (a)	10
			(b)	11
3	Brothers	Hemophiliac	7 (a)	14
			(b)	14

The clinical manifestations of the hemorrhagic tendencies in these carriers are very interesting. All but four of the carriers had a subjective history of very easy bruising. One mother had a severe hemorrhage after an operation and required 2 pints of blood. Two mothers had profuse bleeding after tonsillectomy which lasted 3 days in both instances. Two other mothers had a history of prolonged slow bleeding after tooth extractions. As would be expected, none had any bleeding difficulties associated with delivery of their children. The three carriers who showed normal AHG levels had no evidence of any hemorrhagic tendency.

Of the 3 women who had normal AHG levels by this test, two were definite carriers and the third was a probable carrier by the definitions previously established.

DISCUSSION

A deficiency of AHG in the female has been a concept that has only gradually been accepted. This probably stems from the classic description of hemophilia occurring only in the male and never in the female. However, there now appear to be 3 distinct entities where the female shows lowered levels of antihemophilic globulin.

First, the human female with classic AHG deficiency is a rare occurrence but has been described several times.[14-17] This results from the mating of a hemophiliac male and a carrier female with the female offspring deriving an affected gene from each parent.

Second, vascular hemophilia which is characterized by a prolonged bleeding time with normal platelets, usually a positive Rumple-Leede test, anatomically abnormal capillaries, and a low level of plasma AHG has been well characterized in the female.[18-25] The importance of careful differential diagnosis in this regard has been recently stressed by Graham.[26]

The third, a partial deficiency of AHG in carriers, is a subject of debate. Some authors have found that these women have no bleeding tendencies evident clinically,[5, 6] whereas others have described definite bleeding tendencies as evidenced by severe and prolonged bleeding after tooth extractions[8, 27, 28] and tonsillectomies,[27, 28] easy bruising,[27, 29, 30] and menorrhagia.[29, 30] In 20 of 26 (77 per cent) of our carriers there was a subjective history of bruising easily while 3 of 26 (11.5 per cent) had prolonged bleeding after surgical operation and 2 of 26 (7 per cent) after tooth extractions.

Until recently, the only carriers with proved low AHG levels have been isolated cases who had levels of AHG low enough to cause bleeding tendencies and to be demonstrable with the laboratory techniques available.[27, 28, 31] Otherwise the studies performed on carriers of severe hemophilia have produced normal results.[4, 5, 8, 10] However, occasionally an abnormal subject has appeared with enough regularity that the search to demonstrate a difference from the normal has continued. Margolius and Ratnoff[6] had 4 carriers out of 27 with a prolonged silicone clotting time. Also, 1 carrier had an abnormal modified thromboplastin generation time and a prolonged recalcification time when the patient's plasma was added to hemophilic plasma. Merskey and MacFarlane[8] described 3 carriers of 33 who had slightly prolonged clotting times and prothrombin consumption less than normal. Didisheim and co-workers[29] in a large series of carriers of both AHG and plasma thromoplastin component deficiency showed that 12.7 per cent of AHG carriers and 28.6 per cent of plasma thromboplastin component carriers were abnormal in two or more of their coagulation study tests. In addition, Stefanini and Dameshek[32] have described the presence of the alpha X globulin in the plasma electrophoretic pattern of hemophiliacs and in many carriers.

Recently there have been two major contributions in the endeavor to test for carrier states. Pitney and Arnold[7] demonstrated an AHG level below normal in 10 carriers and 4 possible carriers of AHG deficiency in 25 patients tested by a modified thromboplastin generation test. These observations are strongly supported by the findings in our series. The carrier state in another type of hemophilia was demonstrated by Bond and associates[33] who have described a definite deficiency of plasma thromboplastin component in carriers of plasma thromboplastin component deficiency by using a new substitute for the platelet factor in the thromboplastin generation test.

These previous positive studies have utilized methods other than the erythrocytin test for determining these AHG levels. The extreme sensitivity of this erythrocytin test is shown by the ease which it detects the addition of 0.0008 ml. of normal plasma to 1 ml. of hemophilic plasma. Although the mechanism of this reaction is not known, the observation remains that as increasing minute amounts of normal plasma are added to the test substrate, the prothrombin consumption values increase. Conversely, the prothrombin consumption values do not change with the addition of AHG-deficient hemophilic plasma.

The reproducibility of results with this test is shown by the similar prothrombin consumptions noted in the family studies. The data so obtained in Fig. 3 indicate almost identical levels throughout 3 generations in one family. The similarity of results obtained in siblings and their offspring are shown in Table II. Reproducibility is also indicated by observing the same prothrombin consumption in replicate determinations in the same subject. These tests were done without identification as to prior clinical diagnosis.

It seems evident from these studies that the female carriers manifest an abnormal X chromosome by a lowered plasma AHG activity. The exact content is probably determined by an abnormal gene that is transmitted from generation to generation. However, the ability to diagnose the carrier state is still not available. It is to be noted that although the vast majority of these carriers showed abnormally low AHG levels by this method, three developed normal AHG plasma activity.

SUMMARY

Twenty-one definite carriers and five probable carriers of antihemophilic globulin (AHG) deficiency were assayed for the AHG plasma levels by means of a modification of the erythrocytin method of Quick. Twenty-three of the subjects showed abnormally low AHG levels (30 to 40 per cent) in the range between those found in normal persons and in patients with hemophilia.

The similar levels of AHG in siblings and in different generations of the same family have been illustrated, which suggest that the exact plasma level of AHG is evidently a genetically controlled factor.

Five of the mothers had hemorrhagic difficulties associated with surgical procedures and twenty-two gave a personal subjective history of easy bruising.

REFERENCES

1. Hay, J.: Account of a Remarkable Hemorrhagic Disposition Existing in Many Individuals of the Same Family, N. England J. Med. & Surg. **2**: 221, 1813. Quoted by Quick, A. J.: Hemorrhagic Diseases, Philadelphia, 1957, Lea & Febiger, p. 135.
2. Schloessmann, H.: Die Hämophilie, Neue deutsche Chirugie, Stuttgart, 1930, Ferdinand Enke.
3. Skold, E.: On Hemophilia in Sweden and Its Treatment by Blood Transfusion, Acta med. scandinav. Supp. **150**: 4, 1944.
4. Biggs, R., and Douglas, A. S.: The Thromboplastin Generation Test, J. Clin. Path. **6**: 23, 1953.
5. Gardikas, C., Katsiroumbas, P., and Kottas, C.: The Antihemophilic Globulin Concentration in the Plasma of Female Carriers of Haemophilia, Brit. J. Haemat. **3**: 377, 1957.
6. Margolius, A., Jr., and Ratnoff, O. D.: A Laboratory Study of the Carrier State in Classic Hemophilia, J. Clin. Invest. **35**: 1316, 1956.
7. Pitney, W. R., and Arnold, B. J.: Plasma Antihaemophilic Factor (AHF) in Families of Patients With Hemorrhagic States, Brit. J. Haemat. **5**: 184, 1959.
8. Merskey, C., and MacFarlane, R. G.: The Female Carrier of Hemophilia, Lancet **1**: 487, 1951.
9. Graham, J. B., Collins, D. L., Jr., Godwin, I. D., and Brinkhous, K. M.: Assay of Plasma Antihemophilic Activity in Normal, Heterozygous (Hemophilia) and Prothrombopenic Dogs, Proc. Soc. Exper. Biol. & Med. **77**: 294, 1951.
10. Shinowara, G. Y.: Enzyme Studies on Human Blood. XI. The Isolation and Characterization of Thromboplastic Cell and Plasma Components, J. Lab. & Clin. Med. **38**: 11, 1951.
11. Quick, A. J., Georgatsos, J. G., and Hussey, C. V.: The Clotting Activity of Human Erythrocytes, Am. J. M. Sc. **228**: 207-213, 1954.
12. Georgatsos, J. G., Hussey, C. V., and Quick, A. J.: Nature of Action of New Clotting Factor Obtained From Erythrocytes, Am. J. Physiol. **181**: 30, 1955.
13. Quick, A. J.: Hemorrhagic Diseases, Philadelphia, 1957, Lea & Febiger, pp. 408-420.
14. Taylor, K., and Biggs, R.: A Mildly Affected Female Haemophiliac, Brit. M. J. **1**: 1494, 1957.
15. Merskey, C.: The Occurrence of Haemophilia in the Human Female, Quart. J. Med. **20**: 299, 1951.
16. Quick, A. J., and Hussey, C. V.: Hemophiliac Condition in the Female, J. Lab. & Clin. Med. **42**: 929, 1953. (Abst.)
17. Israels, M. C. G., Lempert, H., and Gilbertson, E.: Haemophilia in the Female, Lancet **1**: 1375, 1951.
18. Matter, M., Newcomb, T. F., Melly, A., and Finch, C. A.: Vascular Hemophilia. The Association of a Vascular Defect With a Deficiency of Antihemophilic Globulin, Am. J. M. Sc. **232**: 421, 1956.
19. Nilsson, I. M., Blomback, M., and Francken, I. V. : On an Inherited Autosomal Hemorrhagic Diathesis With Antihemophilic Globulin (AHG) Deficiency and Prolonged Bleeding Time, Acta med. scandinav. **159**: 35, 1957.
20. Van Creveld, S., Jordan, F. L. J., Punt, K., and Veder, H. A.: Deficiency of Antihemophilic Factor in a Woman, Combined With a Disturbance in Vascular Function, Acta med. scandinav. **151**: 381, 1955.
21. Nilsson, I. M., Blomback, M., Jorpes, E., Blomback, B., and Johansson, S.: V. Willebrand's Disease and Its Correction With Human Plasma Fraction 1-0, Acta med. scandinav. **159**: 179, 1957.
22. Alexander, B., and Goldstein, R.: Dual Hemostatic Defect in Pseudohemophilia, J. Clin. Invest. **32**: 551, 1953. (Abst.)
23. De Vries, S. I., Bosman, M. T., and Smiers, C. M. T.: Deficiency of Anti-hemophilic Globulin in Women, Associated With a Prolonged Bleeding Time, Acta Haemat. **21**: 206, 1959.
24. Verstraete, M., and Vanderbroucke, J.: Diathèses hémorragiques et activité du facteur antihémophilique chez la femme, Rev. hémat. **10**: 665, 1955.
25. Larrieu, M. J., and Soulier, J. P.: Déficit en facteur antihémophilique A chez une fille, associé à un trouble du saignement, Rev. hémat. **8**: 361, 1953.
26. Graham, J. B.: The Inheritance of Vascular Hemophilia: A New and Interesting Problem in Human Genetics, J. M. Educ. **34**: 385, 1959.
27. Douglas, A. S., and Cook, I. A.: Deficiency of Antihemophilic Globulin in Heterozygous Haemophilic Females, Lancet **2**: 616, 1957.
28. Fantl, P., and Margolis, J.: Alpha-Prothromboplastin Deficiencies (Hemophilia) of Differing Degrees in a Mother and Son, Brit. M. J. **1**: 640, 1955.

29. Didisheim, P., Ferguson, J. H., and Lewis, J. H.: Hemostatic Data in Relatives of Hemophiliacs A and B, A. M. A. Arch. Int. Med. **101:** 347, 1958.
30. Graham, J. B., McLeodon, W. W., and Brinkhous, K. M.: Mild Hemophilia an Allelic Form of the Disease, Am. J. M. Sc. **225:** 46, 1953.
31. Wilkinson, J. F., Israels, M. C. G., Nour-Eldin, F., and Turner, R. L.: Unusual Transmission of the Haemophilic Gene, Brit. M. J. **2:** 1528, 1957.
32. Stefanini, M., and Dameshek, W.: The Hemorrhagic Disorders, New York, 1955, Grune & Stratton, Inc., p. 269.
33. Bond, T., Celander, D. R., and Guest, M. M.: The Detection of Plasma Thromboplastin Component (PTC) Deficiency Carriers, Fed. Proc. **18:** 51, 1959.

CHANGES IN BILE CANALICULI PRODUCED BY NORETHANDROLONE: ELECTRON MICROSCOPIC STUDY OF HUMAN AND RAT LIVER

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Norethandrolone is a substituted testosterone which produced cholestasis in some patients. It was given in doses known to produce sulfobromophthalein retention to patients with normal liver function and to normal rats to see if ultrastructural changes occurred which would clarify the mechanism of jaundice development. Liver biopsy specimens were obtained from all subjects and, by histologic examination with the light microscope, these were shown to be normal. Under the electron microscope, many bile canaliculi in all patients and animals were dilated and their microvilli were shortened and reduced in number, similar to the alterations encountered in cholestasis. The remainder of the liver cell was normal. This suggests that norethandrolone exerts a specific effect on bile canaliculi and that jaundice from administration of this drug is not the result of hypersensitivity.

NORETHANDROLONE, 17-ethyl nortestosterone, a weak androgen with potent nitrogen sparing ability, produced jaundice in a few underweight patients[1, 2] to whom it was given because of its protein anabolic action. Sulfobromophthalein retention, however, was noted[3] in almost all patients receiving the drug, and this developed regularly within two weeks if the dose was large enough.[4] The jaundice was usually transient and associated with elevated serum activity of alkaline phosphatase and of glutamic oxalacetic transaminase.[2] Liver biopsy specimens from the jaundiced patients showed cholestasis usually without inflammatory exudate in the portal tracts. In an attempt to elucidate the mechanism of this jaundice, norethandrolone was given to a group of patients and to rats in doses known to produce retention of sulfobromophthalein, and specimens of liver tissue obtained by needle biopsy were examined under the electron microscope.

MATERIAL AND METHODS

Four male patients received orally 90 mg. norethandrolone daily in divided doses for a period of 2 weeks. They initially had normal liver function as measured by routinely used tests. One was recovering from a subtotal gastrectomy for duodenal ulcer, one had an esophagogastrectomy for carcinoma of the esophagus, one had a pancreatectomy for nonobstructing tumor, and one had severe functional dysphagia. All were underweight

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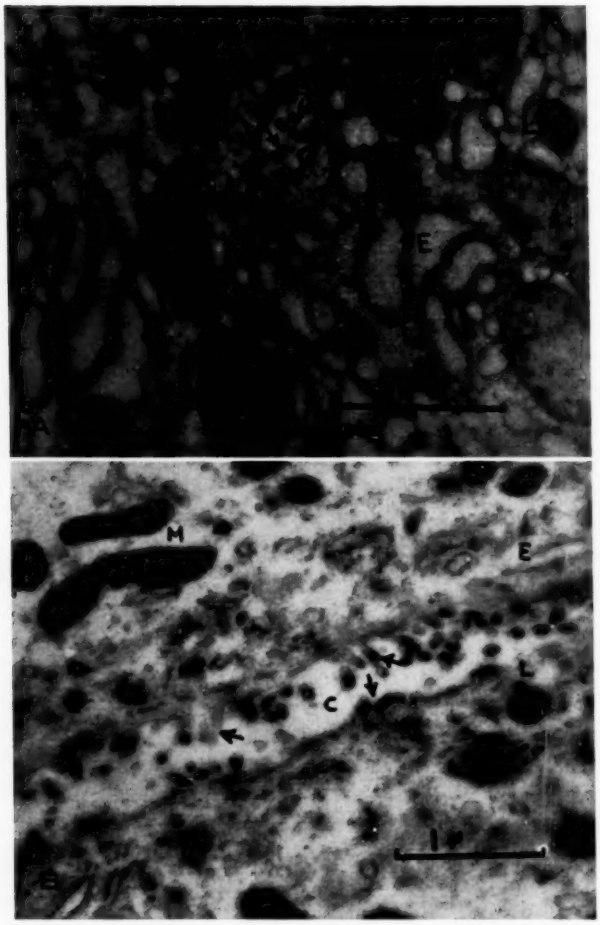

Fig. 1.—*A*, Electron micrograph of normal rat liver showing undilated canaliculus *(C)* with microvilli (arrows) lying between two cells and formed from cell walls *(CW)* of the two cells. Mitochondria *(M)*, ergastoplasm *(E)*, and lysosomes *(L)* are normal. (Magnification ×45,000; reduced ½.) *B*, Electron micrograph of normal human liver showing longitudinal section of undilated bile canaliculus *(C)* with many microvilli (arrows). Normal organelles such as mitochondria *(M)*, lysosomes *(L)*, and ergastoplasm *(E)* are seen in the cell cytoplasm. (Magnification ×40,000.)

and ambulatory. Liver biopsy was performed at the end of the treatment with the Menghini needle.

Small bits of the fresh tissue were quickly placed in ice cold 2 per cent osmium tetroxide (unbuffered and titrated to pH 7.4). After fixation for 60 to 90 minutes, the tissue was washed, dehydrated in alcohol, and then embedded in *n*-butyl methacrylate. Sections 0.1 μ in thickness were cut in the Porter Blum microtome. They were mounted on coated copper grids and examined with a Philips model EM100 electron microscope at 100 kilovolts.

The rest of the specimens were fixed in formalin for routine histologic examination. In addition, two rats, each weighing 200 grams, received intramuscular daily injection of 12.5 mg. norethandrolone in sesame oil with benzyl alcohol for 8 days. At the end of this time the animals were stunned by a blow on the head, and a biopsy specimen of liver

tissue was obtained from the animal, quickly cut into small pieces, placed in osmium tetroxide, and prepared as was the human material.

RESULTS

The rat liver tissue appeared normal on routine histologic examination. The human livers showed mild nonspecific changes commensurate with the ages of the patients, their state of nutrition, and their underlying illness or recent operation. These changes included mild portal infiltration with inflammatory cells, slight proliferation of bile ductules surrounded by inflammatory cells, a few areas of focal necrosis, and an increase in the size and number of Kupffer

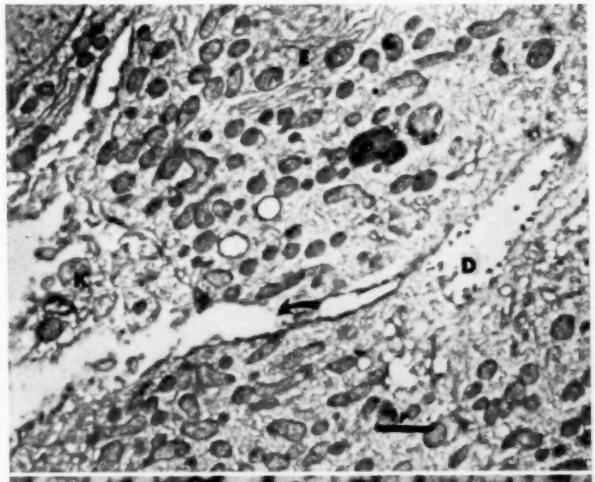

Fig. 2.

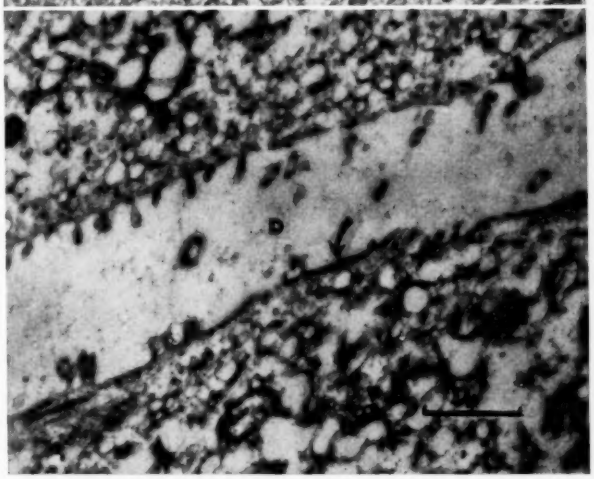

Fig. 3.

Fig. 2.—Electron micrograph of human liver after administration of norethandrolone showing dilated bile canaliculus *(D)* near extension of tissue space (arrow) between two cells. The ergastoplasm *(E)* and the numerous ovoid mitochondria are normal. A fragment of a Kupffer cell (K) is seen on the left. (Magnification ×15,000; reduced ½.)

Fig. 3.—Electron micrograph of longitudinal section of dilated bile canaliculus *(D)* between two parenchymal cells in liver of patient treated with norethandrolone. Note the segments which are bare of microvilli (arrow). (Magnification ×30,000; reduced ½.)

cells. These varied from lobule to lobule, and not all were present in each patient. Considerable lipofuscin pigment was noted in the centrolobular zone. No evidence of bile stasis was encountered, although in some areas the bile canaliculi appeared widened when viewed with the oil immersion objective.

Under the electron microscope the structure of the individual liver cells was normal both in the human and rat livers. Nuclei, nucleoli, ergastoplasm, mitochondria, and cell borders seemed to be well preserved. In rat livers amorphous material containing no organelles and presumed to be glycogen was abundant (Fig. 5). In the human livers vacuolated osmiophilic bodies varying from 0.5 to 2.0 μ in diameter were seen and thought to be lipofuscin granules. No other lipid accumulations were found.

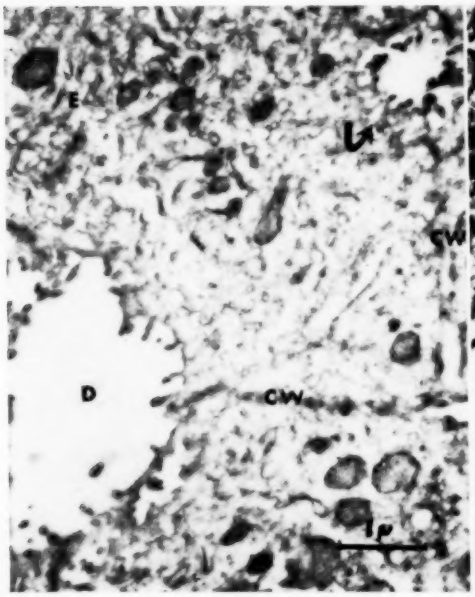

Fig. 4.

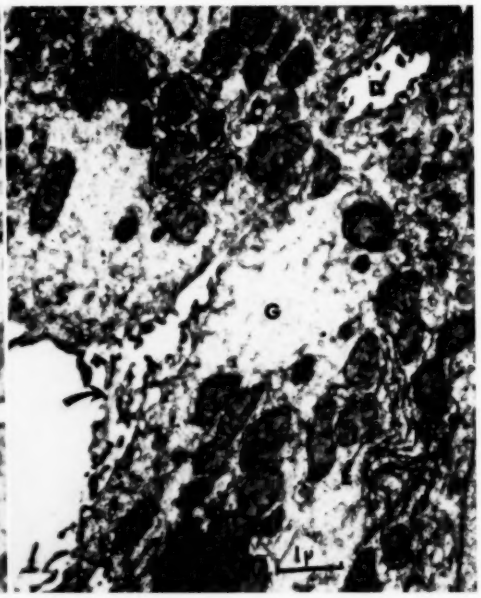

Fig. 5.

Fig. 4.—Same specimen as Fig. 3 showing cross section of two canaliculi draining one liver cell, one dilated *(D)* with short and thin microvilli and the other (arrow) with normal microvilli. The walls of the liver cell *(CW)* and the ergastoplasm *(E)* are normal (Magnification ×25,000; reduced ½.)

Fig. 5.—Dilated bile canaliculus *(D)* in a rat given norethandrolone 12.5 mg. intramuscularly daily for 8 days with much glycogen *(G)*, normal ergastoplasm *(E)*, and ovoid mitochondria. In the extension of the tissue space a few fine reticulum fibers (arrow) are noted. (Magnification ×20,000; reduced ½.)

The bile canaliculi showed the most striking changes. Both in the rat and human livers, one fourth to one third of the canaliculi were dilated from a normal diameter of less than one micron (Fig. 1) to 2 or 3 μ (Figs. 2-5). Dilated canaliculi were sometimes within 5 μ of undilated ones and, indeed,

occasionally bordered on the same liver cell (Fig. 4). The organelles in the liver cells which formed these dilated canaliculi were normal. Bile plugs were not found.

The microvilli in the undilated canaliculi were about 0.1 μ in diameter and 0.5 μ in length. In the round cross sections of canaliculi 10 to 25 microvilli could be counted. Some dilated canaliculi had normal microvilli as far as number, size, and shape were concerned, whereas others contained fewer, thinner, and shorter microvilli. In addition, microvilli were missing from stretches of canaliculi as long as 1.0 μ.

DISCUSSION

An alteration of the canalicular membrane of the liver cells is the morphologic substrate of human cholestasis as seen under the electron microscope.[5] In all cases of jaundice associated with cholestasis, the microvilli of the bile canaliculi were stunted or missing, although some bile canaliculi were dilated and others were not. This change might be explained as the result of increased luminal pressure in extrahepatic biliary obstruction and in some forms of intrahepatic cholestasis, whereas in others in which no evidence for increased pressure existed, the canalicular changes were considered to be primary lesions.

Administration of norethandrolone in doses known to produce retention of sulfobromophthalein was associated with canalicular dilatation and changes of microvilli in the absence of cholestasis, at least as reflected in serum bilirubin levels. A mechanical cause for canalicular alteration cannot be demonstrated. Inflammatory lesions in the portal tracts (so-called cholangiolitis) which can be demonstrated by light microscopy in some instances of drug induced jaundice, for instance, in stages of chlorpromazine induced jaundice,[6] are not necessarily present in patients who develop jaundice after administration of norethandrolone and were absent in the cases under discussion. Moreover, the lack of uniformity of the dilatation militates against elevated intraluminal pressure as the cause of the alteration of the canaliculi. The normal appearance of the liver cells themselves even under the electron microscope speaks against a connection between damage of the cytoplasm of the liver cells and the canalicular changes. The latter therefore have to be considered as a primary process involving the canalicular membrane of the liver cells. The relation of the lesion to cholestasis requires further investigation. Widening of the bile canaliculi and reduction of the microvilli should reduce the resistance to the flow of the viscous bile rather than increase it. Nevertheless, further study of the membrane function reflected by the presence of microvilli is therefore required.

Quantitative rather than qualitative differences in the bile canaliculi between cholestatic jaundice which occurs sporadically and the changes which occur regularly even without jaundice in man and animals after administration of norethandrolone suggest that the same mechanism underlies both. This implies that the cholestatic type of jaundice which follows administration of substituted testosterone preparations is a regular response with variations in intensity.

SUMMARY

Norethandrolone in doses known to produce retention of sulfobromophthalein was given to four patients and two rats, and specimens of liver were examined under the electron microscope. The characteristic alteration was dilatation of some bile canaliculi to two or three times normal diameter with shortening or loss of microvilli in these canaliculi. The canalicular changes were related to the cholestasis sporadically produced by this drug. They seem to represent a milder degree of the same process which suggests that cholestatic jaundice caused by norethandrolone is not a hypersensitivity reaction.

REFERENCES

1. Dunning, M. F.: Jaundice Associated With Norethandrolone (Nilevar) Administration, J. A. M. A. **167**: 1242, 1958.
2. Schaffner, F., Popper, H., and Chesrow, E.: Cholestasis Produced by the Administration of Norethandrolone, Am. J. Med. **26**: 249, 1959.
3. Kory, R. C., Bradley, M. H., Watson, R. N., Callahan, R., and Peters, B. J.: A Six-Month Evaluation of an Anabolic Drug, Norethandrolone, in Underweight Persons. II. Bromsulphalein (BSP) Retention and Liver Function, Am. J. Med. **26**: 243, 1959.
4. Heaney, R. P., and Whedon, G. D.: Impairment of Hepatic Bromsulphalein Clearance by Two 17-Substituted Testosterones, J. Lab. & Clin. Med. **52**: 169, 1958.
5. Schaffner, F., and Popper, H.: Electron Microscopic Study of Human Cholestasis, Proc. Soc. Exper. Biol. & Med. **101**: 777, 1959.
6. Popper, H., and Schaffner, F.: Pathology of Jaundice Resulting From Intrahepatic Cholestasis, J. A. M. A. **169**: 1447, 1959.

SERUM COMPLEMENT IN AMYLOIDOSIS

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Serum complement levels are studied in a group of 24 patients with various forms of amyloidosis. Included in this group are 10 patients with lepromatous leprosy and amyloidosis, 7 patients with amyloidosis and bone marrow plasmacytosis, the majority of whom had multiple myeloma, and 7 patients with amyloidosis without concomitant marrow plasmacytosis. No uniform depression or elevation was noted in the serum complement levels in the group as a whole; however, in the patients with amyloid and marrow plasmacytosis, a significant depression of serum complement occurred. A control group of patients with multiple myeloma and lymphoma, who had marrow plasmacytosis without associated amyloid disease, showed no depression in serum complement levels. The possible significance of this observation with respect to an in vivo complement fixation by amyloid is discussed in the light of what is currently known concerning kinetics of complement and antibody production.

DURING the course of a study of serum complement variations in patients with connective tissue disorders,[1] we encountered a patient with amyloidosis secondary to malignant lymphoma who showed no detectable serum complement in five determinations over a period of 11 months. If deposition of amyloid represents part of an abnormal immune process, absence of serum complement in such patients could indicate in vivo complement fixation by an antigen-antibody reaction. Abnormally low or absent complement might reflect deviations of synthesis of serum complement or abnormal losses through rapid turnover within the body. We therefore elected to study other patients with various forms of amyloid disease.

METHODS AND MATERIALS

Serum complement was determined by the procedure of Fischel[2] after the method of Mayer and collaborators[3, 4] using the 50 per cent hemolysis technique which estimates complement activity by hemolysis of sensitized sheep red cells. Calculation of complement in 50 per cent units per milliliter of serum has been previously outlined.[1] All sera studied had been separated and stored at -40° C. within 2 hours of collection. Sera that showed low or absent complement activity were tested for possible anticomplementary action by mixing with serum of normal complement activity, diluted appropriately, and subsequent complement determination in the usual manner.

PLAN OF STUDY

Twenty-four patients with amyloidosis were studied. These patients were divided into 3 groups.

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Group 1 comprised a series of 10 patients with far advanced lepromatous leprosy and presumed secondary amyloidosis.* Patients were included in this group of presumed amyloid subjects if Congo red retention showed progressive increase above 70 per cent, or in the face of Congo red retention below 70 per cent, if hepatosplenomegaly and persistent albuminuria were present. Patients in the leprosy-amyloid group were studied together with 60 patients who had leprosy of similar duration and extent who did not manifest the signs of amyloidosis.

Group 2 included 7 patients with significant bone marrow plasmacytosis (10 per cent or greater). Of these, 5 patients had multiple myeloma and associated amyloidosis, one patient had malignant lymphoma, and one had amyloidosis of secondary distribution, which was due to unknown cause. Diagnosis of amyloidosis were confirmed by autopsy or biopsy in all 7 patients. A control group of 10 patients with myeloma and lymphoma who were known to have marrow plasmacytosis but who did not have amyloidosis were studied.

Group 3 constituted 7 patients with amyloidosis of various types without concomitant marrow plasmacytosis. Amyloid deposition was proved by biopsy or autopsy in all patients of this group.

RESULTS

Complement levels on 123 normal subjects previously reported[1] were used as controls. Range of normal was from 35 to 54 50 per cent hemolytic units per milliliter of serum (U50 per cent) with a mean normal of 44 U50 per cent ± 1 standard deviation of 4.7.

In the leprosy group, serum complement in 10 subjects with amyloidosis ranged between 36 and 66 U50 per cent with mean value of 52 U50 per cent. This represented no significant difference from a control group of 60 patients with leprosy alone whose mean and median serum complement level was 52 U50 per cent.

In the group of patients with amyloidosis and marrow plasmacytosis, serum complement ranged between 0 and 34 U50 per cent with a mean value of 11 U50 per cent. A control group of 10 patients with plasmacytosis, but without amyloid disease (6 myeloma and 4 lymphoma) showed a range of serum complement between 37 and 70 U50 per cent, with a mean value of 56 U50 per cent.

The 7 patients with other forms of amyloidosis, but without plasmacytosis showed serum complements ranging between 34 and 82 U50 per cent, with a mean of 53 U50 per cent. Results are tabulated for all groups studied in Table I.

All of the 24 patients with amyloidosis had electrophoretic analyses performed. No relationship could be noted between elevations in gamma globulin and depressions of serum complement activity. There was no evidence of significant anticomplementary activity in the sera from the various patients with amyloidosis studied.

DISCUSSION

Uniform depression of serum complement does not occur in the patients studied with various forms of amyloidosis. The one group of patients who did exhibit complement depression with concomitant amyloidosis were those who showed a relative plasmacytosis in the bone marrows. Most of this latter group

*Furnished through the assistance of the U. S. Public Health Service Hospital at Carville, La.

TABLE I

PATIENT NO.	SERUM COMPLEMENT (U50%/ML. SERUM*)	REMARKS
Group 1. Amyloidosis and Leprosy (10 Patients).—		
1	66	Control leprosy alone (60 patients)
2	36	Range 40-75 U50%
3	48	Mean 52 U50%
4	42	
5	56	
6	48	
7	58	
8	51	
9	54	
10	62	
	mean 52	
Group 2. Amyloidosis and Plasmacytosis.—		
1	34	Control plasmacytosis alone (10 patients)
2	0	Range 37-70 U50%
3	0	Mean 56 U50%
4	12	
5	7	
6	0	
7	27	
	mean 11	
Group 3. Amyloidosis Without Plasmacytosis.—		
1	70	
2	82	
3	57	
4	57	
5	38	
6	34	
7	38	
	mean 53	

*Range of normal serum complement levels is 35 to 54 U50 per cent ± 1 S.D. of 4.7.

had multiple myeloma. Marrow plasmacytosis alone was not accompanied by a depression of complement level, as can be noted among the control patients with myeloma studied. These data suggest that complement kinetics—possibly synthesis, utilization, or excretion—in amyloidosis associated with plasmacytosis is of different character from that of the other patients with amyloidosis studied. Moreover, complement in patients with amyloidosis and plasmacytosis might be fixed in vivo by a quantitatively greater amount of antigen-antibody interaction than in the remainder of subjects with amyloidosis. A serum inhibitor, not demonstrable by the usual crude anticomplementary tests, may explain low complement values in the group with amyloid and marrow plasmacytosis. Such an inhibitor has been recently described by Levy and Lepow.[5]

The role of plasma cells in the genesis of amyloidosis has been mentioned by Teilum[6] during the experimental production of amyloid using nitrogen mustard. He observed a suppression of plasma cells and other pyronin-positive cells in the course of induced amyloid deposition. Plasma cells have been closely linked with antibody production in numerous studies[7-9] but never specifically with complement variation. Indeed, there is reason to suspect that the factors governing complement production or actual serum complement level may be of a

different sort from those related to antibody production. In support of this concept, studies by Schwab and co-workers[10] have demonstrated that when x-radiation prevented antibody production in rabbits, serum complement levels did not fall. However, these authors did notice close temporal relationships between depression of serum complement and disappearance of antigen from the circulation. Later, the appearance of homologous antibody in the experimental animals was correlated with return of serum complement to normal levels and the development of tissue lesions. This concept suggests an active role for complement in the genesis of cellular damage during experimental antigen-antibody reaction.

Low complement levels have been previously attributed to liver disease[11, 12] by appraisal of patients and by animal studies. In carefully reviewing available autopsy findings and serial laboratory studies among the 24 patients studied, no predominance of hepatocellular damage or hepatic amyloid deposition was apparent in those patients who showed low complement values. Similarly, no specific locus of organ involvement by amyloid deposition could be correlated with the low serum complement levels found among our patients. This was somewhat surprising, since many of the patients with amyloidosis studied were considered to have the nephrotic syndrome, a condition previously often associated with low complement levels.[13, 14]

Since no uniform depression of serum complement occurs in the spectrum of amyloid disease studied here, speculation as to the binding of complement by an in vivo antigen-antibody reaction seems unwarranted, though not impossible. It would appear more likely that patients with amyloidosis and myeloma or a myeloma-like disease have some deficiency in complement production or replacement. Studies are indicated to test complement production and turnover in such patients, as in their responses to acute infections or other similar well-recognized stimuli of immune responses.

SUMMARY

1. Serum complement levels in 24 patients with amyloidosis were studied.

2. The amyloidosis of lepromatous leprosy, chronic suppurative disease, and other disease states was not accompanied by either uniform elevation or depression of serum complement levels.

3. All 7 patients with plasmacytosis and amyloidosis showed low serum complement levels.

4. Plasmacytosis in bone marrow alone, without amyloid deposition, was not accompanied by complement depression.

5. When amyloidosis is suspected, low serum complement levels accompanied by marrow plasmacytosis may help to appraise the diagnosis.

We are indebted to Sister Hilary Ross of the U. S. Public Health Service Hospital, Carville, La., for the sera studied from patients with leprosy. We should also like to express our appreciation to Dr. Evan Calkins of the Arthritis Unit, Massachusetts General Hospital, who furnished us sera and encouragement during the course of this study.

REFERENCES

1. Williams, R. C., Jr., and Law, D. H., IV: Serum Complement in Connective Tissue Disorders, J. LAB. & CLIN. MED. **52**: 273, 1958.
2. Fischel, E. E., Pauli, R. H., and Lesh, J.: Serological Studies in Rheumatic Fever. II. Serum Complement in the Rheumatic State, J. Clin. Invest. **28**: 1172, 1949.
3. Mayer, M. M., Eaton, B. B., and Heidelberger, M.: Spectrophotometric Standardization of Complement and the Activating Effect of Magnesium and Other Cations on the Hemolytic Process, J. Exper. Med. **84**: 535, 1946.
4. Kabat, E. A., and Mayer, M. M.: Experimental Immunochemistry, Springfield, Ill., 1958, Charles C Thomas, Publisher.
5. Levy, L. R., and Lepow, I. H.: Assay and Properties of Serum Inhibitor of C' 1-Esterase, Proc. Soc. Exper. Biol. & Med. **101**: 608, 1959.
6. Teilum, G.: Studies on Pathogenesis of Amyloidosis. II. Effect of Nitrogen Mustard in Inducing Amyloidosis, J. LAB. & CLIN. MED. **43**: 367, 1954.
7. Fagraeus, A.: Antibody Production in Relation to the Development of Plasma Cells, Acta med. scandinav. (Suppl. 204) **130**: 3, 1948.
8. Moeschlin, S., and Demiral, B.: Antikörperbildung der Plasma zellen in Vitro, Klin. Wchnschr. **30**: 827, 1952.
9. Keuning, F. J., and van der Slikke, L. B.: The Role of Immature Plasma Cells, Lymphoblasts, and Lymphocytes in the Formation of Antibodies, as Established in Tissue Culture Experiments, J. LAB. & CLIN. MED. **36**: 167, 1950.
10. Schwab, L., Moll, F. C., Hall, T., Brean, H., Kirk, M., Van Zandt Hawn, C., and Janeway, C. A.: Experimental Hypersensitivity in the Rabbit. Effect of Inhibition of Antibody Formation by X-radiation and Nitrogen Mustards on the Histologic and Serologic Sequences and on the Behavior of Serum Complement, Following Single Large Injections of Foreign Proteins, J. Exper. Med. **91**: 505, 1950.
11. Jordan, F. L. J.: Significance of Complement Test in Liver Diseases With Jaundice, Acta med. scandinav. **144**: 268, 1953.
12. Rice, C. E., Duhamel, L., and Girard, A.: The Effect of Prolonged Injection of Ethionine on Rabbits and Guinea Pigs Fed a High Cholesterol Diet. I. On Complement Titer, Canad. J. Comp. Med. & Vet. Sc. **19**: 167, 1955.
13. Fischel, E. E., and Gajdusek, D. C.: Serum Complement in Acute Glomerulonephritis and Other Renal Diseases, Am. J. Med. **12**: 190, 1952.
14. Lange, K., Graig, F., Oberman, J., Slobody, L., Ogur, G., and LoCastro, F.: Changes in Serum Complement During the Course and Treatment of Glomerulonephritis, A. M. A. Arch. Int. Med. **88**: 433, 1951.

ANTIBODY RESPONSE IN PATIENTS WITH ACUTE LEUKEMIA

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WITH THE TECHNICAL ASSISTANCE OF ROBERT KOLB, JULIUS A. KASSEL, AND CLARENCE SZWED

The clinical course of patients with acute leukemia is characterized by frequent and severe infections. During a study of factors of host resistance to infection in ten patients with acute leukemia, the antibody responses to 5 antigens were measured and compared to those of ten normal control subjects. An attempt was made to determine the relationship between the degree of antibody response and the frequency of bacterial infections observed in the individual patient. Because of their possible relation to antibody production and infection, serum proteins were also studied in both leukemic and normal subjects.

The response to any one antigen did not differ significantly in the 2 groups, and all but one of the patients with acute leukemia responded to at least one antigen. A significantly poorer response in the patients with acute leukemia could be demonstrated only by comparing the over-all antibody response to all antigens. Factors which may have influenced antibody production in the patients, including antimetabolite therapy and deficient caloric intake, are discussed. The degree of antibody response could not be related to the initial level or subsequent change in serum gamma globulin concentration. There was no correlation between the degree of antibody response and the frequency of bacterial infection in the individual patient.

THE natural history of the leukemias is characterized by frequent and severe infection.[1-3] That this is related to impaired humoral defense mechanisms against infection has been suggested.[4, 5] Although there have been a number of studies pertaining to the antibody response in the chronic leukemias, such studies in patients with acute leukemia have been few and in apparent conflict. Larson and Tomlinson[6] reported that nine of eleven patients with acute leukemia had higher than normal antibody responses to pneumococcal polysaccharide, whereas Kibrick[7] observed a diminished antibody response to polyvalent influenza vaccine and to formalin fixed pneumococcal vaccine. Additional studies to resolve these reported differences may be of aid in understanding the mechanisms of host defense against infection, and may be of further interest because of current attention to problems of immunity in relation to bone marrow transplantation in acute leukemia. Furthermore, other aspects of the nature of

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the antibody response in patients with acute leukemia, not previously investigated, are worthy of consideration. These include the variability in response to different test antigens within the same patient, and the question of whether or not there is indeed a correlation between the degree of antibody response to test antigens and the frequency of infection in the individual patient.

The present work was undertaken during a study of factors of host resistance to infection in patients with acute leukemia. The antibody responses to a number of challenge antigens were measured and compared to those of normal subjects after inoculation with identical antigens. Because of their possible relation to antibody production, serum proteins were also studied during the inoculation period in both leukemic and normal subjects. In this article the results of these investigations are reported.

METHODS AND MATERIALS

The antibody responses of ten patients with acute leukemia were compared to those of ten normal control subjects. Certain factors were employed in choosing both the leukemic and the normal subjects. Except for Patients 1 and 2 (Table I), individuals who gave a history of inoculation with any of the antigens employed, or of an illness possibly related to one of these antigens in a 3 year period preceding the study were not selected. The control subjects (Table I) included one patient with petit mal epilepsy who was otherwise healthy (Control 11), and nine healthy volunteers (Controls 12 to 20), three of whom were nurses (Controls 18 to 20). Because of the possible effect of steroid therapy on antibody production, those patients who required cortisone or related drugs on admission or during the study period of 26 days were excluded. Only two patients initially included in the study were subsequently eliminated because of steroid administration. The antibody study was begun in the leukemic patients at the onset of chemotherapy for acute leukemia. Drugs used were 6-mercaptopurine and 4-amino-N^{10} methyl pteroylglutamic acid (Methotrexate). The bone marrow criteria used to classify the activity of the disease were those accepted by the Clinical Studies Panel of the Cancer Chemotherapy National Service Center.[8] The detailed clinical observations and the methods employed to determine the nature of the infections encountered in the patients with acute leukemia have been recorded in another publication.[1]

The antigens employed were typhoid-paratyphoid vaccine,* influenza vaccine† (type A, Conley strain), mumps vaccine, diphtheria toxoid, and tetanus toxoid. Each milliliter of the typhoid-paratyphoid vaccine contained 1,000 million killed typhoid bacteria, 250 million killed paratyphoid A, and 250 million killed paratyphoid B bacteria. The mumps vaccine and the influenza vaccine were prepared from the allantoic fluid of embryonated hens' eggs infected with the respective viruses and inactivated with formalin.

The following immunization procedure was employed. On days 1, 13, and 26, approximately 30 ml. of blood was obtained from each patient and from each control subject. The blood specimens were centrifuged and the sera were immediately frozen for subsequent antibody determinations and for electrophoretic analyses. The mumps and the typhoid-paratyphoid antigens were administered subcutaneously. The tetanus toxoid, the diphtheria toxoid, and the influenza vaccine were given intramuscularly. Separate needles and syringes were used for each antigen. Each injection was made at a different site. The immunization schedule was: day 1, mumps vaccine, 1.0 ml., diphtheria toxoid, 0.5 ml., tetanus toxoid, 0.5 ml.; day 2, influenza vaccine, 1.0 ml., typhoid-paratyphoid vaccine, 0.5 ml.; day 8, mumps vaccine, 1.0 ml.; day 9, typhoid-paratyphoid vaccine, 0.5 ml.; day 16, typhoid-paratyphoid vaccine, 0.5 ml.; day 18, diphtheria toxoid, 0.5 ml., tetanus toxoid, 0.5 ml.

*All the antigens except the influenza vaccine were commercial preparations obtained from Lederle Laboratories.

†Obtained from Parke, Davis & Company.

TABLE I. ANTIBODY DETERMINATIONS IN 10 PATIENTS WITH ACUTE LEUKEMIA AND IN 10 NORMAL CONTROL SUBJECTS ON DAYS 1, 13, AND 26

SUBJECT			TYPE LEU-	TYPHOID* H			MUMPS*			INFLUENZA*			DIPHTHERIA ANTITOXIN (μ/ML.)			TETANUS ANTITOXIN (μ/ML.)		
NO.	AGE	SEX	KEMIA	1	13	26	1	13	26	1	13	26	1	13	26	1	13	26
Patients.—																		
1	3	M	L	<20	20	20	<8	<8	<8	<10	<10	<10	3.0	16.000	20.000	3.250	>3.250	>13.000
2	5	F	L	<20	<20	<20	<8	<8	<8	80	80	80	4.0	4.000	6.000	10.400	8.000	10.400
3	7	F	L	<20	<20	<20	<8	<8	<8	20	<10	<10	1.0	0.250	2.000	0.400	0.400	1.400
4	8	M	L	<20	40	20	<8	64	16	80	1,280	1,280	0.005	32.000	32.000	0.060	>30.000	>30.000
5	22	M	L	80	160	160	<8	<8	<8	20	20	40	0.020	16.000	16.000	<0.004	13.000	8.000
6	34	F	L	<20	20	40	<8	8	32	<10	<10	<10	0.050	13.000	16.000	0.005	0.062	0.031
7	16	F	G	<20	<20	<20	<8	<8	<8	160	160	1,280	0.200	1.500	6.000	0.002	0.002	0.016
8	28	F	G	80	80	320	<8	<8	<8	<10	<10	<10	0.008	0.003	0.003	0.008	0.005	0.005
9	47	M	G	20	240	240	<8	<8	<8	<10	40	40	0.320	16.0	32.0	<0.001	<0.001	0.008
10	47	M	G	80	160	80	<8	<8	<8	<10	<10	<10	1.000	5.000	4.000	0.004	0.004	0.004
Control Subjects.—																		
11	11	F		<20	20	40	<8	16	16	40	160	640	0.010	4.000	4.000	0.016	7.000	7.000
12	18	F		<20	40	40	<8	16	16	40	160	320	0.200	12.000	8.000	0.125	14.100	14.100
13	19	M		<20	40	40	<8	32	32	<10	2,560	640	0.010	2.000	1.500	0.002	0.002	0.008
14	20	M		<20	20	40	<8	<8	<8	20	160	160	0.005	0.040	1.000	0.002	0.002	0.004
15	20	M		<20	40	80	<8	<8	<8	20	320	160	0.010	10.000	8.000	0.001	0.001	0.003
16	20	F		<20	20	40	<8	16	16	40	80	40	1.0	24.0	24.0	0.002	0.002	0.002
17	21	M		20	80	40	<8	16	16	80	160	80	0.050	64.0	64.0	0.001	0.001	0.004
18	31	F		40	40	80	<8	<8	<8	<10	160	320	1.0	32.0	28.0	0.016	4.0	4.0
19	38	F		80	80	80	<8	16	16	<10	160	640	0.005	0.040	1.0	0.500	8.0	16.0
20	49	F		320	320	320	<8	16	16	<10	80	160	0.005	0.01	0.02	0.016	1.5	2.0

*Expressed as reciprocal of highest serum dilution.

The rapid slide test was utilized for measuring typhoid antibodies.[9] Hemagglutination inhibition was used to test for antibodies against influenza.[10] Mumps antibody was measured by the complement-fixation technique of Bengtson.[11] The diphtheria antitoxin titers[12] and the tetanus antitoxin titers[13] were determined according to standard methods. The three sera from a patient were tested simultaneously for the presence of a single antibody.

Analytic paper electrophoresis and total serum protein determinations by the biuret technique were performed as described elsewhere.[14] Based on data from 44 normal subjects in this laboratory, the normal gamma globulin was found to be 1.19 Gm. per cent with a standard deviation of 0.27 Gm. per cent.[15] Using 1.19 Gm. per cent as normal, the gamma globulin determinations from the patients and the normal control subjects were expressed in terms of the per cent of mean normal.

After analyzing the degree of antibody responses on day 13 and on day 26 (as shown in Table I) with a number of different statistical methods, a fourfold or greater rise was considered a "significant" antibody response to antigenic stimulation. Based on this definition, a "+" or a "0" was marked for each antibody response to each antigen, and the total antibody score for patients and control subjects was determined (Table II).

RESULTS

Table I records each patient's age, sex, and type of leukemia, the age and sex of the control subjects, and the antibody titers obtained on days 1, 13, and 26. The rank t test[16] shows no significant difference in the range of preinoculation antibody titers (day 1) for each of the 5 antibody groups between patients with leukemia as compared to the corresponding antibody group in the control subjects. From the standpoint of over-all antibody synthesis (sum of horizontal rows, Table II) the group with leukemia responded less well to challenge antigens than did the normal control group, for the rank t test shows a t^2 value of 7.35 which is significant at the 1 per cent level.

The difference in response to each antigen can be compared by examining the vertical columns in Table II. Of the group of patients with leukemia, three responded to typhoid vaccine, two to mumps vaccine, and three to influenza vaccine. In comparison, seven control subjects responded to typhoid vaccine, seven to mumps vaccine, and eight to influenza vaccine. Of the ten patients with leukemia seven developed significant rises after administration of diphtheria toxoid in contrast to all ten of the control subjects. Six patients and seven normal subjects had significant titer rises after they received tetanus toxoid. In spite of these suggestive differences, the chi square test with Yates' correction factor[17] shows no significant statistical difference between the number of patients with leukemia who responded to a particular antigen as compared to the number of control subjects.

The responses between the two groups did not differ primarily in magnitude, for those patients who responded to a given challenge antigen did so to a degree usually equal to and occasionally greater than that of the normal subjects. (Compare in Table I the diphtheria and tetanus titers of the leukemic and control group on day 26.) Moreover, the antibody titers obtained on day 13 (Table I) show that those leukemic patients who responded to the challenge antigens did so as promptly as the normal subjects.

Antibody Production Depending Upon Type of Leukemia and Age.—The

TABLE II. FOURFOLD OR GREATER RISE ON DAY 26 IN PATIENTS WITH LEUKEMIA AND IN NORMAL SUBJECTS

SUBJECT NO.	TYPHOID H	MUMPS	INFLUENZA	DIPHTHERIA	TETANUS	INDIVIDUAL SCORE
Patients.—						
1	0	0	0	+	+	2
2	0	0	0	0	0	0
3	0	0	0	0	+	1
4	0	+	+	+	+	4
5	0	0	0	+	+	2
6	+	+	0	+	+	4
7	0	0	+	+	+	3
8	+	0	0	0	0	1
9	+	0	+	+	0	3
10	0	0	0	+	0	1
Totals	3	2	3	7	6	21
Control Subjects.—						
11	+	+	+	+	+	5
12	+	+	+	+	+	5
13	+	+	+	+	+	5
14	+	0	+	+	0	3
15	+	0	+	+	0	3
16	+	+	0	+	0	3
17	+	+	0	+	+	4
18	0	0	+	+	+	3
19	0	+	+	+	+	4
20	0	+	+	+	+	4
Totals	7	7	8	10	7	39

mean antibody scores were found for patients with lymphocytic and with granulocytic leukemia and for patients under and over the age of 15 years. Patients with lymphocytic leukemia had a slightly higher antibody score, 2.2, than did patients with granulocytic leukemia, whose mean score was 1.8. Patients over the age of 15 years had a score of 2.2 as compared to 1.8 for patients under the age of 15 years.

Antibody Production and Serum Proteins.—Measurements of the serum gamma globulin levels, summarized in Table III, indicate no significant difference between the pre- and postinoculation levels of gamma globulin in either the leukemic or the normal group. Furthermore, there is no significant correlation between the degree of antibody response and changes in gamma globulin in either group. In addition, there was no correlation between the capacity to form antibody and the serum albumin or total serum globulin. Similar lack of correlation between albumin and globulin levels and the ability to form antibody has been noted by Balch[18] and Larson and Tomlinson.[19]

Nutritional Factors and Antibody Production.—In view of the different opinions expressed,[18-20] regarding the significance of nutritional deficiency resulting from starvation or from debilitating disease on antibody production, the caloric and protein intake of six patients and of six normal subjects was examined. These are listed in Table III. During the antibody study the daily caloric intake of six of ten patients with acute leukemia ranged from approximately 1,000 to 2,000 calories per day, with an average intake of 1,400 calories.

The daily protein intake ranged from 30 to 70 Gm. with an average of 51 Gm. The mean antibody score (six patients) was 1.8. For the six normal subjects, the daily caloric intake ranged from approximately 1,300 to 3,800 calories with an average intake of 2,700 calories. The protein intake ranged from 40 to 134 Gm. with an average intake of 93 Gm. The mean antibody score (six control subjects) was 3.8.

It is interesting that within each of the two groups there was no clear relationship between nutritional intake and antibody production.

Antibody Response and Relationship to Infection.—In an effort to evaluate the significance of the antibody response as it related to infection, the antibody score for each patient was compared with the number of infections that each patient had while under observation in the Clinical Center with active acute leukemia. There were seventeen bacterial infections which occurred in eight of the ten patients. Two patients had no bacterial complications during the time they were observed. The major clinical diagnoses were: septicemia without a demonstrable primary source of infection (6 cases), pyelonephritis (5 cases), pharyngitis (3 cases), cellulitis (2 cases), and pneumonia (1 case). The causative organisms were *Escherichia coli* (6 instances), *Micrococcus pyogenes* var. *aureus,* coagulase positive (5 instances), *Pseudomonas aeruginosa* (2 instances), *Micrococcus pyogenes* var. *albus,* coagulase negative (2 instances), Group A beta *hemolytic streptococci* (1 instance), and *Klebsiella sp.* (1 instance). Since the number of proved bacterial infections and the number of days in the hospital varied for each patient, the number of proved infections per 100

TABLE III. ANTIBODY SCORE, GAMMA GLOBULIN CHANGES, AND NUTRITIONAL FACTORS IN PATIENTS WITH ACUTE LEUKEMIA AND IN NORMAL CONTROL SUBJECTS

PATIENT OR CONTROL NO.	ANTIBODY SCORE	GAMMA GLOBULIN LEVELS (% OF MEAN NORMAL)*		AVERAGE DAILY INTAKE IN 6 PATIENTS AND 6 NORMAL SUBJECTS	
		DAY 1	DAY 26	CALORIES	PROTEINS (GM.)
1	2	149	129	1,284	50
2	0	85	70	1,352	49
3	1	148	97	1,001	30
4	4	110	91	——	—
5	2	67	46	1,986	71
6	4	108	108	——	—
7	3	177	187	1,638	62
8	1	104	108	——	—
9	3	103	167	1,170	41
10	1	106	108	——	—
				1,407 average	51
11	5	44	57	——	—
12	5	57	70	1,986	64
13	5	80	86	2,991	102
14	3	97	93	2,821	95
15	3	111	87	3,794	134
16	3	118	113	1,347	40
17	4	126	154	3,312	123
18	3	108	108	——	—
19	4	94	92	——	—
20	4	129	161	——	—
				2,709 average	93

*Mean "normal" gamma globulin = 1.19 Gm. per cent ± 0.27 Gm. per cent.

TABLE IV. ANTIBODY SCORE AND PROVED BACTERIAL INFECTIONS IN 10 PATIENTS WITH ACUTE LEUKEMIA

PATIENT NO.*	NO. DAYS OBSERVED WITH ACTIVE DISEASE	NO. OF PROVED BACTERIAL INFECTIONS	NO. OF INFECTIONS PER 100 HOSPITAL DAYS	NO. OF DAYS WITH INFECTION	% OF DAYS WITH INFECTION	ANTIBODY SCORE
9	92	5	5.4	52	56.5	3
5	46	3	6.5	10	21.7	2
4	138	3	2.2	25	18.1	4
1	120	1	0.8	11	9.2	2
8	190	2	1.1	15	7.9	1
6	54	1	1.9	4	7.4	4
7	100	2	2.0	5	5.0	3
2	58	0	0.0	0	0.0	0
3	78	0	0.0	0	0.0	1
10	44	0	0.0	0	0.0	1

*Arranged in descending order of percentage of days with infection.

hospital days is used as an expression of rate. Analysis of the data in Table IV indicates there is no correlation between the antibody score and the number of infections per 100 hospital days or the per cent of days with infection.

DISCUSSION

This study indicates that, after the administration of the antigens employed, the antibody response of a group of patients with acute leukemia was significantly less than that of a group of normal subjects. Nevertheless, it is to be noted that the patients with leukemia were capable of achieving some degree of antibody response. Thus, all patients except one had a fourfold antibody rise after the administration of at least one of the antigens. Except for tetanus toxoid, there was no significant statistical difference in frequency of response to the four other antigens between the two groups. That a statistical difference did exist in the over-all antibody response, but not in the frequency of response to each antigen, may reflect the fact that the former represents the response accumulated from each of the 5 antigens. Thus, a statistical difference may be expected to appear in the total score, before one is observed in the score for each antigen.

The use of 5 antigens in this study was predicated on the assumption that there may be a significant difference in the degree of response to different antigens within the same individual. Use of a single antigen which may give rise to a small amount of antibody, or conversely, a potent antigen which yields a large amount of antibody, may lead to erroneous conclusions regarding the over-all antibody potential of the patient. The concept that, in disease as well as in health, variations may be found in response to different types of antigenic stimulation, may resolve some of the seemingly conflicting reports in the earlier literature in which only one antigen, without adequate controls, was employed in the test system.

It is to be emphasized that a number of diverse factors could have affected the antibody score reported for these patients because of the difficulties in attempting to control a study such as this. All the patients with acute leukemia

received folic acid and purine antagonists during the period of testing for antibody response. Although this was not desirable from a "study standpoint," it was nevertheless unavoidable because effective therapy cannot be withheld from patients with acute leukemia for as long as 30 days. Ludovici and Axelrod[21] have shown that rats maintained on a diet deficient in folic acid have impaired hemagglutinin production in response to inoculation with human red blood cells. When 6-mercaptopurine is given simultaneously with bovine albumin to rabbits, antibody responses are profoundly suppressed.[22] The fact that the patients with acute leukemia responded as they did despite antimetabolite therapy suggests, at least, that their immunologic apparatus is not severely impaired. As could be anticipated, the caloric and protein intake were significantly lower in the acute leukemia group as compared to the control group. In the leukemic group, caloric intake appeared to be related to the general condition of the patient and within this group there was no correlation between dietary intake and antibody production. Four of the ten patients with leukemia were 8 years of age or younger, whereas all of the control subjects were 11 years of age or older. Except to reduce the total amount of data, however, the exclusion of the four patients in the lower age group would not significantly affect the results or interpretation of antibody response in the remaining patients. By design, patients who required steroids on admission to the Clinical Center or during the antibody study period were not included in this evaluation. Antibody production in these patients could have been different from that in those who were not treated with steroids and who therefore were less acutely ill. Finally, although the rank t test suggested that the prior antigenic experiences for the leukemic and normal groups were similar, that this was actually the case cannot be categorically stated. Even though the contribution of each of the factors mentioned cannot be stated with certainty, it is clear that mechanisms other than deficient antibody production are responsible for the frequency of infections observed in acute leukemia for no correlation existed between antibody production and frequency of infection in the patients with leukemia.

Significance of Antibody Response in Relation to Infection in Patients With Acute Leukemia.—In view of the not infrequent attempts to attribute infections in patients with leukemia[4, 5] and lymphoma[23, 24] to impaired antibody responses, it is important to state some of the theoretical objections to these earlier reports, and, indeed, to our own. Most of the antigen-antibody systems used in these clinical studies were selected because of technical considerations pertaining to the availability and administration of the antigens, and facility for measuring the resulting antibodies. It cannot be inferred that the antibodies measured in this or in other studies are in themselves, the products of *the* essential factors in the mechanism of immunity or in the prevention of infection. Moreover, there is no necessary connection between the serum level of antibody and the degree of tissue responsiveness upon which active immunity largely depends.[25] On the other hand, the absence of circulating antibodies or the inability to find a close association between a high antibody titer and degree of resistance is inadequate evidence that specific antibodies have not been formed, since antibody may

become readily fixed to tissue cells. Yet the number and the invasive characteristics of an infecting agent at a susceptible site may be such as to overcome even a high degree of specific tissue immunity. Therefore, there is no unequivocal statement that can be made regarding the significance of an observed response to a given challenge antigen. There is no decisive answer to the question of whether or not specific antibodies are always concerned in bacterial immunity, or if there are some other more important defense mechanisms of the tissues which function independently of them. At best, measurements of antibodies in response to challenge antigens which may not be important in clinical situations can be regarded only as an index of immunity. The clinical observations made during this study lend credence to these thoughts, for there was no correlation between the frequency of clinical infection and the degree of antibody response to the antigens employed.

SUMMARY

1. The antibody responses to 5 antigens were measured in ten patients with acute leukemia and these responses were compared to those of ten normal control subjects.

2. The response to any one antigen did not differ significantly in the two groups and all but one of the patients with acute leukemia responded to at least one antigen.

3. A significantly poorer response in the patients with acute leukemia could be demonstrated only by comparing the over-all antibody rises to all antigens.

4. It is possible that antimetabolite administration and deficient caloric intake were responsible for the difference which existed between the leukemic and control groups. Within the leukemic group, however, there was no correlation between dietary intake and antibody production.

5. The degree of antibody response could not be related to the initial level or subsequent change in serum gamma globulin concentration.

6. There was no correlation between the degree of antibody response and the frequency of bacterial infection within the individual patient.

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REFERENCES

1. Silver, R. T., Utz, J., Frei, E., III, and McCullough, N.: Fever, Infection and Host Resistance in Acute Leukemia, Am. J. Med. **24**: 25, 1958.
2. Kirschbaum, J. D., and Preuss, F. S.: Leukemia: A Clinical and Pathological Study of One Hundred and Twenty-Three Fatal Cases in a Series of 14,400 Necropsies, Arch. Int. Med. **71**: 777, 1943.
3. Forkner, C.: Leukemia and Allied Diseases, New York, 1938, The Macmillan Company, pp. 22 and 23.
4. Rotky, H.: Concerning the Ability of the Leukemic to Form Antibodies, Zentralbl. f. innere Med., Leipz. **35**: 953, 1914.
5. Howell, K. M.: The Failure of Antibody Formation in Leukemia, Arch. Int. Med. **26**: 706, 1920.
6. Larson, D. L., and Tomlinson, L. J.: Quantitative Antibody Studies in Man. III. Antibody Response in Leukemia and Other Malignant Lymphomata, J. Clin. Invest. **32**: 317, 1953.

7. Kibrick, S., cited in Kushner, M., and Thomas, L.: Infection in Disseminated Neoplastic Disease, Bull. New York Acad. Med. **33**: 684, 1957.
8. Clinical Studies Panel, Cancer Chemotherapy National Service Center: Criteria for the Evaluation of Response to Treatment in Acute Leukemia (Letter to the Editor), Blood **11**: 676, 1956.
9. Welch, H., and Stuart, C. A.: A Rapid Slide Test for the Serologic Diagnosis of Typhoid and Paratyphoid Fevers, J. LAB. & CLIN. MED. **21**: 411, 1936.
10. American Public Health Association: Diagnostic Procedures for Virus and Rickettsial Diseases, ed. 2, New York, 1956, The Association, pp. 241-262.
11. Bengtson, I. A.: Complement Fixation in Rickettsial Diseases, Pub. Health Rep. **59**: 402, 1944.
12. Frobisher, M.: *in* Diagnostic Procedures and Reagents, ed. 3, New York, 1950, American Public Health Association, pp. 184-186.
13. Wadsworth, A. B.: Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, ed. 3, Baltimore, 1947, Williams & Wilkins Company, p. 635.
14. Fahey, J. L., McCoy, P. F., and Goulian, M.: Chromatography of Serum Proteins in Normal and Pathologic Sera, J. Clin. Invest. **37**: 272, 1958.
15. Fahey, J. L.: Unpublished data.
16. Bross, I. D.: Is There an Increased Risk? Fed. Proc. **13**: 815, 1954.
17. Mainland, D.: Elementary Medical Statistics, Philadelphia, 1952, W. B. Saunders Company, p. 95.
18. Balch, H. H.: Relation of Nutritional Deficiency in Man to Antibody Production, J. Immunol. **64**: 397, 1950.
19. Larson, D. L., and Tomlinson, L. J.: Quantitative Antibody Studies in Man. II. The Relation of the Level of Serum Proteins to Antibody Production, J. LAB. & CLIN. MED. **39**: 129, 1952.
20. Wohl, M. G., Remgold, J. G., and Rose, S. B.: Antibody Response in Patients With Hypoproteinemia, Arch. Int. Med. **83**: 402, 1949.
21. Ludovici, P. P., and Axelrod, A. E.: Circulating Antibodies in Vitamin Deficiency States, Proc. Soc. Exper. Biol. & Med. **77**: 526, 1951.
22. Schwartz, R., Stack, J., and Dameshek, W.: Effect of 6-Mercaptopurine on Antibody Production, Proc. Soc. Exper. Biol. & Med. **99**: 164, 1958.
23. Schier, W. W.: Cutaneous Anergy and Hodgkin's Disease, New England J. Med. **250**: 353, 1954.
24. Dubin, I. N.: The Poverty of the Immunological Mechanism in Patients With Hodgkin's Disease, Ann. Int. Med. **27**: 898, 1947.
25. Wilson, C. S., and Miles, A. A.: Principles of Bacteriology and Immunity, ed. 4, Baltimore, 1955, Williams & Wilkins Company, Vol. 2, p. 1290.

EFFECT OF PLASMIN IN VITRO ON CLOTTING FACTORS IN PLASMA

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Plasmin is a proteolytic enzyme of plasma capable of digesting fibrin and certain other proteins in vitro at or near neutrality. Conclusions regarding its effect on clotting factors considered to function prior to the conversion of fibrinogen to fibrin have been conflicting. The results of this study demonstrate that proaccelerin, antihemophilic factor, and Christmas factor activities were markedly decreased after incubating normal plasma with plasmin. Hageman factor, plasma thromboplastin antecedent, Stuart factor, and precursor of serum prothrombin conversion accelerator activities were unaltered in such an incubation mixture.

Incubation of streptokinase with normal plasma, on the other hand, resulted primarily in a loss of proaccelerin activity. This observation is consistent with the concept that full activation of plasmin by streptokinase does not occur in unclotted plasma. Occasionally, small losses of antihemophilic factor and Christmas factor activities were found. No losses of Hageman factor or proconvertin complex activities were observed. Streptokinase could be shown to inactivate prothrombin only after a prolonged incubation period.

THE fibrinolytic activity of plasmin, a proteolytic enzyme of plasma, has suggested its use in thromboembolic states. Necessarily, considerable attention has been paid to other biologic effects of this enzyme. The possibility that plasmin may digest clotting factors other than fibrinogen and fibrin has been investigated repeatedly, but with conflicting results. The present study was designed to test the effect of plasmin in vitro upon those clotting factors thought to function prior to the conversion of fibrinogen to fibrin. The results of these studies suggest that plasma, treated with plasmin, may be deficient in antihemophilic factor, proaccelerin, Christmas factor, and prothrombin. No measurable change was observed in the other factors tested.

NOMENCLATURE AND MATERIALS

The term *plasmin* refers to one or more proteolytic enzymes of plasma capable of digesting fibrinogen, fibrin, and certain other proteins in vitro at, or near, neutrality. This enzyme has also been called *fibrinolysin*. It is present in plasma in the form of an inactive precursor, *plasminogen*.

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Plasminogen was extracted from a lyophilized preparation of Cohn Fraction III of human plasma* by Kline's[1] method. This preparation contained about 100 Christensen[2] units per milliliter after activation with streptokinase. Prior to its use, three parts of plasminogen was incubated with one part of streptokinase for 10 minutes at room temperature to achieve activation. The concentrations of streptokinase used ranged from 625 to 7,500 units per milliliter in the reaction mixtures. This preparation will be referred to as *streptokinase activated plasmin.*

Commercial human *plasmin,*† which has been activated with streptokinase during its preparation was dissolved in buffer to provide concentrations ranging from 5,000 to 12,000 Christensen units per milliliter. This will be referred to as *commercial plasmin.*

Streptokinase is a filterable principle found in cultures of hemolytic streptococci which is capable of activating human plasminogen to plasmin. Streptokinase (high purity)‡ was dissolved in buffer in concentrations ranging from 2,500 to 30,000 units per milliliter.

Normal human plasma was prepared from blood drawn with syringes coated with silicone (G-E Dri-Film) and needles coated with tris-(2 hydroxyethyl) dodecyl ammonium chloride.§ The blood was mixed with one ninth its volume of 0.13M trisodium citrate solution and centrifuged at 2,500 r.p.m. for 15 minutes at room temperature. The supernatant plasma was stored at -25° C. for as long as 3 months before use. Plasmas were also prepared from the blood of patients with classic hemophilia (lacking antihemophilic factor), Christmas disease (lacking Christmas factor or plasma thromboplastin component), Hageman trait (lacking Hageman factor), plasma thromboplastin antecedent deficiency,‖ parahemophilia (lacking proaccelerin or factor V), deficiency of precursor of serum prothrombin conversion accelerator, and Stuart factor deficiency.¶

Asbestos absorbed bovine plasma for use in the assay for proconvertin[5] was prepared from bovine blood which had been mixed with one ninth its volume of 0.1M potassium oxalate. The plasma was filtered through 20 per cent asbestos filter pads** in a Seitz filter and stored at -25° C.

Charcoal adsorbed bovine plasma[6] was prepared by filtering oxalated bovine plasma through powdered hardwood charcoal†† three times.

Rabbit brain *thromboplastin* (Difco) was suspended in a 0.15M solution of sodium chloride in a concentration of 37.5 mg. per milliliter. A crude "*cephalin*" preparation, which contained material insoluble in acetone but soluble in chloroform, was prepared from rabbit brain by the method of Bell and Alton[7] and was stored at -25° C. This extract was diluted with 14 parts of buffer before use. *Russell's viper venom*‡‡ was prepared in a 1:100,000 dilution in buffer. Equal amounts of these dilutions of crude cephalin and Russell's viper venom were mixed prior to use in the assay for Stuart factor.

Barium sulfate adsorbed oxalated beef plasma§§ was mixed with an equal volume of human oxalated serum prepared according to Alexander's method[8] to give a suitable reagent for use in the assay for true prothrombin content.

---

*Lot No. 1420, obtained from Dr. T. D. Gerlough, E. R. Squibb & Sons, New Brunswick, N. J.

†Lot No. 1108-86-M, obtained from Merck Sharp & Dohme, West Point, Pa.

‡Obtained from Dr. J. Ruegsegger, Medical Research Section, Lederle Laboratories, Pearl River, N. Y.

§Monocote, The Armour Laboratories, Chicago, Ill.

‖This patient was studied through the courtesy of Drs. I. S. Wright and Margaret Todd, New York City, and had been studied by Dr. Robert Rosenthal in New York, who believes that the laboratory findings are consistent with mild plasma thromboplastin antecedent deficiency.[4]

¶I am indebted to Dr. William Krivit, Department of Pediatrics, University of Minnesota School of Medicine, for the gift of this plasma.

**John C. Carlson, Ltd., Ashton U. Lyne, Lancashire, England.

††Springfield Facing Company, Willimansett, Mass.

‡‡Stypven, Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

§§Prothrombin-free beef plasma, Hyland Laboratories, Los Angeles, Calif.

The buffer used in this study consisted of 7.30 Gm. of sodium chloride, 2.76 Gm. of barbital, and 2.06 Gm. of sodium barbital diluted to a volume of 1 L. with distilled water. The pH of the mixture was 7.4.

METHODS

The effect of streptokinase activated plasmin, commercial plasmin, or of streptokinase alone upon clotting factors was tested in the following manner. The preparation to be tested was incubated with human plasma. This plasma was usually obtained from normal individuals. However, in experiments to determine the effect of streptokinase activated plasmin upon plasma thromboplastin antecedent, plasma from a patient deficient in Hageman factor was used. After the preparation to be tested had been incubated with plasma for an appropriate interval, the concentrations of clotting factors in the mixture were measured.

1. *Incubation of Enzyme With Plasma.*—A mixture of 1.0 ml. of a solution of streptokinase activated plasmin, commercial plasmin or streptokinase and 1.5 ml. of normal plasma was incubated at 37° C. for 30 or 60 minutes in Pyrex tubes with an internal diameter of 11 mm. The tubes were then transferred to a 4° C. bath and their contents immediately diluted twenty to three hundred twenty fold with cold buffer. These dilutions were kept at 4° C. until the concentrations of clotting factors in the mixtures were measured.

As a control, 1.0 ml. of the solution of plasmin or streptokinase was incubated at 37° C. for the same period. At the end of this time, this solution was transferred to a 4° C. bath and mixed with 1.5 ml. of plasma which had been simultaneously incubated at 37° C. This mixture was then diluted with cold buffer in the manner described. This control tested the influence of plasmin or of streptokinase on the assays for clotting factors.

An additional control mixture which contained no enzyme consisted of 1.5 ml. of plasma diluted with 1.0 ml. of buffer and was incubated at 37° C. for 30 or 60 minutes. At the end of the incubation period, this mixture was chilled to 4° C. and appropriately diluted with cold buffer. This buffer control tested the influence of incubation on the clotting factors measured.

The residual clotting factor activity is expressed as the percentage of that remaining in the control tube which contained enzyme, if notable discrepancies existed between the two control tubes.

2. *Clotting Factor Assay Techniques.*—a. The concentration of antihemophilic factor in the diluted mixtures was measured by testing their ability to shorten the recalcified clotting time, measured at 25° C., of hemophilic plasma by previously described methods.[9] Comparison of the corrective effects of treated and untreated plasma allowed an estimation of the degree to which antihemophilic factor was inactivated by treatment with plasmin or streptokinase. Similarly, the concentrations of Christmas factor, plasma thromboplastin antecedent, and Hageman factor were determined by measuring the effect of the mixtures on the recalcified clotting time of plasma deficient in the respective factor.

b. The proaccelerin (factor V) and precursor of serum prothrombin conversion accelerator content of the various mixtures was determined by testing their ability to correct the one stage prothrombin time,[10] measured at 37° C., of plasma known to be deficient in the factor to be measured.[11] In several instances, normal oxalated plasma which had been stored at -25° C. for 3 months or more was substituted for genetically deficient plasma in measuring proaccelerin.

c. Stuart factor was measured by the method of Sise and associates.[12] The mixture to be tested was added to charcoal filtered bovine oxalated plasma, and the recalcified clotting time was then measured in the presence of Russell's viper venom and crude cephalin at 37° C. Prior to their use in the assay, the reagents were tested against the plasma of a patient believed to be deficient in Stuart factor. This method is though to be insensitive to variations in amounts of prothrombin, proaccelerin, and precursor of serum prothrombin conversion accelerator.

d. The quality of the proconvertin complex (precursor of serum prothrombin conversion accelerator and Stuart factor) which remained in the mixtures at the end of incubation was

determined at 37° C. according to the method of Owren and Aas.[13] This method is thought to be sensitive to changes both in precursor of serum prothrombin conversion accelerator and Stuart factor which are not distinguished, but is insensitive to changes in prothrombin and proaccelerin.

e. The concentration of prothrombin was measured by Alexander's modification of the method of Owren.[8] This technique measures prothrombin in the presence of adequate amounts of proaccelerin and the proconvertin complex.

All assays were done in duplicate in Pyrex tubes with an internal diameter of 8 mm. The results to be described are based on three or more experiments with each type of enzymatic activity with the exception of observations on plasma thromboplastin antecedent and proconvertin. On these two factors, only the effect of streptokinase treated Kline plasminogen was tested. Plasmin and streptokinase were not grossly inactivated during the period of incubation. This was shown by the observation that the clots which formed during the subsequent assays for clotting factors lysed quickly in the presence of plasmin or streptokinase. In contrast, clots formed in the absence of these enzymes did not lyse during the period of observation.

RESULTS

A. *Effect of the Addition of Streptokinase Activated Plasmin to Plasma.*—When normal human plasma was incubated at 37° C. for 60 minutes with streptokinase activated plasmin derived from a Kline plasminogen preparation, 75 to 100 per cent of activity attributable to antihemophilic factor and proaccelerin disappeared (Fig. 1). The decrease in Christmas factor was almost as great. On the other hand, the concentration of prothrombin was either unchanged or, in one of three experiments, diminished only 20 to 25 per cent.

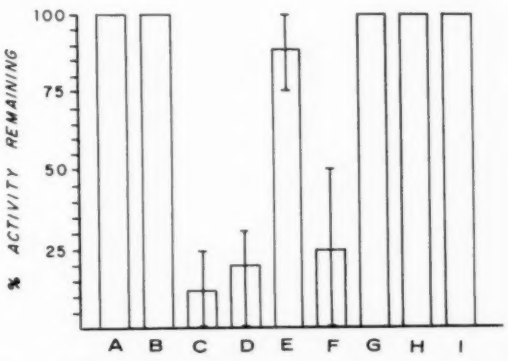

Fig. 1.—Effect of streptokinase activated plasmin on clotting factors in plasma (1 hour incubation period). *A*, Hageman factor. *B*, Plasma thromboplastin antecedent. *C*, Antihemophilic factor. *D*, Christmas factor. *E*, Prothrombin. *F*, Proaccelerin. *G*, Stuart factor. *H*, Precursor of serum prothrombin conversion accelerator. *I*, Proconvertin complex.

No loss of Hageman factor, plasma thromboplastin antecedent, Stuart factor, precursor of serum prothrombin conversion accelerator, or the proconvertin complex was detected. In these experiments, the plasminogen content of the activation mixture was 75 Christensen units per milliliter. The concentration of streptokinase in the activation mixture varied from 625 to 7,500 units per milliliter in different experiments. These differences in streptokinase concentration could not be correlated with loss of clotting factor activity. An example of some of the data obtained in one such experiment is summarized in Table I.

TABLE I. EFFECT OF STREPTOKINASE ACTIVATED PLASMIN ON SOME CLOTTING FACTORS IN NORMAL PLASMA

FACTOR MEASURED	DILUTIONS OF INCUBATION MIXTURES	CLOTTING TIMES	
		TEST MIXTURE (PLASMIN INCUBATED WITH PLASMA)	CONTROL MIXTURE (PLASMIN AND PLASMA INCUBATED SEPARATELY)
Antihemophilic factor	1:20	13 min.	10 min.
	1:80	16 min.	13 min.
	1:∞ (buffer)		16 min.
Christmas factor	1:20	11 min.	9 min.
	1:80	13 min.	12 min.
	1:∞ (buffer)		14 min.
Hageman factor	1:40	12 min.	13 min.
	1:160	19 min.	18 min.
	1:∞ (buffer)		21 min.
Proaccelerin	1:20	58.6 sec.	45.5 sec.
	1:40	64.5 sec.	54.5 sec.
	1:∞ (buffer)		72.0 sec.

In this typical experiment, 0.25 ml. of streptokinase (10,000 U. per milliliter) and 0.75 ml. of Kline plasminogen (100 Christensen units per milliliter) were incubated together at room temperature for 10 minutes to form streptokinase activated plasmin. Then, 1.5 ml. of normal plasma was added, and this test mixture was incubated for 1 hour at 37° C. In the control mixture, the streptokinase activated plasmin and plasma were incubated separately for 1 hour at 37° C. and then mixed.

The concentration of each clotting factor was determined by the methods described. Briefly, the mixtures were diluted serially with buffer prior to assay. They were then tested for ability to correct the defect of plasma lacking the factor to be measured. The activity remaining in the test mixture after incubation may be expressed as a percentage of that contained in the original plasma by comparing the clotting times of similar dilutions of the test mixture and the control.

B. *Effect of the Addition of Commercial Plasmin to Plasma.*—When plasma was incubated at 37° C. for 30 minutes with commercial human plasmin, activity attributable to antihemophilic factor again decreased 75 to 100 per cent, proaccelerin-like activity, 60 to 100 per cent, and Christmas factor activity, 50 to 75 per cent (Fig. 2). Under these circumstances, no change was observed in the activity attributable to prothrombin, Hageman factor, Stuart factor, or the proconvertin complex. The same results were obtained when the concentration of plasmin in the final incubation mixture was varied from 2,000 to 5,000 Christensen units per milliliter.

C. *Effect of the Addition of Streptokinase to Plasma.*—When human plasma was incubated at 37° C. for 60 minutes with streptokinase, the only consistent change observed was a decrease of 50 to 80 per cent in the proaccelerin-like activity (Fig. 3). In addition, in one of five experiments the concentration of Christmas factor and of antihemophilic factor decreased 50 and 25 per cent, respectively. No loss of prothrombin, Hageman factor, or the proconvertin complex was found. Within the range of streptokinase concentrations used (1,000 to 12,500 units per milliliter in final incubation mixtures), there was no correlation between its concentration and degree of proaccelerin inactivation, nor was a large amount used in the experiment in which some inactivation of Christmas and antihemophilic factors occurred.

When mixtures of plasma and streptokinase were incubated for 4 hours instead of 60 minutes, the concentrations of antihemophilic factor, Christmas

factor, and prothrombin all deceased about 50 per cent, and the loss in proaccelerin activity increased to 95 per cent. Even this prolonged period of incubation produced no loss in activity attributable to the proconvertin complex. The effect of this period of incubation on Hageman factor, plasma thromboplastin antecedent, proconvertin, and Stuart factor was not measured.

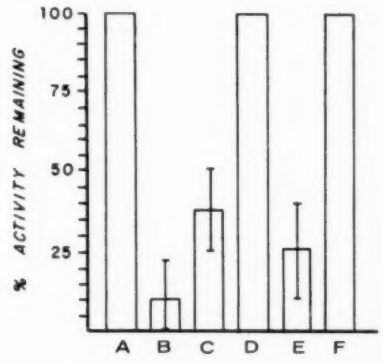

Fig. 2.—Effect of commercial plasmin on clotting factors in plasma (30 minute incubation period). *A*, Hageman factor. *B*, Antihemophilic factor. *C*, Christmas factor. *D*, Prothrombin. *E*, Proaccelerin. *F*, Proconvertin complex.

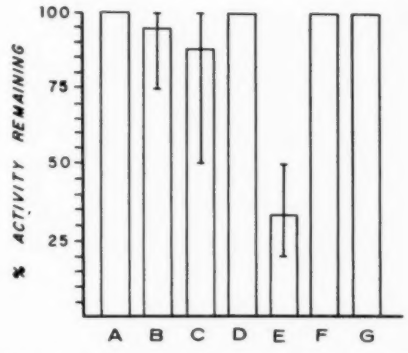

Fig. 3.—Effect of streptokinase on clotting factors in plasma (1 hour incubation period). *A*, Hageman factor. *B*, Antihemophilic factor. *C*, Christmas factor. *D*, Prothrombin. *E*, Proaccelerin. *F*, Stuart factor. *G*, Proconvertin complex.

DISCUSSION

Of the clotting factors studied, those which are relatively more thermolabile appear to be most susceptible to inactivation by human plasmin. The loss of antihemophilic factor activity observed in this study is in agreement with the findings of Alagille and Soulier[14] and Niewiarowski and Latallo[21] in their in vitro studies.

Lewis and co-workers[15] demonstrated the marked instability of a partially purified preparation of proaccelerin when incubated with plasmin. Alagille and Soulier[14] were similarly impressed with the striking effect of plasmin on proaccelerin activity in whole blood, and concluded that of all the clotting factors,

this one was most readily destroyed by plasmin. Marked diminution of proaccelerin activity was also observed in the present study. Proaccelerin was the only factor consistently affected when plasma was incubated with streptokinase for one hour. This observation is consonant with Alagille and Soulier's conclusion. Only prolonged incubation of such a mixture resulted in significant losses of Christmas factor and of antihemophilic factor.

In contrast to the effect of streptokinase alone, streptokinase treated Kline plasminogen or commercial plasmin almost completely inactivated proaccelerin, antihemophilic factor, and Christmas factor within one hour. This difference in behavior was not related to the streptokinase content of the two plasmin preparations. The concentration of the streptokinase in the final incubation mixture which contained streptokinase treated Kline plasminogen was 25 per cent less than in the streptokinase-plasma incubation mixture. Moreover, the amount of Kline plasminogen incubated with 1.5 ml. of plasma was about 75 Christensen units, less than one third the amount in 1.0 ml. of normal human serum.[2] It seems likely, therefore, that the differences observed between the effects of streptokinase and of plasmin were not due to differences in the concentration of either in the incubation mixture. This is in agreement with earlier observations that the addition of streptokinase to unclotted plasma does not fully activate its plasminogen.[16-19]

The pronounced effect of plasmin on prothrombin observed by Seegers and Loomis[20] and Alagille and Soulier[14] was not found in these experiments. Lewis and collaborators[15] have indicated that partially purified prothrombin was resistant to inactivation by plasmin in vitro. The results of the present experiments suggest that prothrombin is inactivated by plasmin at a slower rate than is Christmas factor, antihemophilic factor, or proaccelerin.

The loss of Christmas factor activity in whole blood after incubation in vitro with bovine fibrinolysin was described by Alagille and Soulier.[14]

The lack of effect of plasmin on Hageman factor, plasma thromboplastin antecedent, Stuart factor, and precursor of serum prothrombin conversion accelerator has been a consistent and reproducible finding under the conditions of this study. In contrast, Alagille and Soulier[14] found proconvertin to be somewhat susceptible to proteolysis in vitro.

Under the conditions of these experiments, at least three clotting factors besides fibrinogen in plasma may be readily inactivated by relatively small amounts of plasmin. The use of the activator, streptokinase, on the other hand, produced a less marked, or slower rate of destruction of these susceptible clotting factors. This difference may be of clinical importance.

SUMMARY AND CONCLUSIONS

1. The effects of human plasmin derived from a plasminogen preparation as described by Kline, of commercial human plasmin, and of streptokinase upon the clotting factors in normal plasma were studied in vitro.

2. Proaccelerin, antihemophilic factor, and Christmas factor activities appeared to be most readily lost from plasma upon incubation with plasmin. Only

proaccelerin was readily inactivated upon incubation of plasma with streptokinase. Prothrombin activity was lost in appreciable amounts only after prolonged incubation of plasma with streptokinase, which suggests that it is less readily destroyed by plasmin than are the other three factors.

3. Hageman factor, plasma thromboplastin antecedent, Stuart factor, and precursor of serum prothrombin conversion accelerator (the proconvertin complex) were unaffected in these experiments.

It is a pleasure to acknowledge the invaluable advice and criticisms of Dr. O. D. Ratnoff during these experiments and in the preparation of this manuscript, and the helpful criticisms of Dr. Irvine H. Page in preparing the manuscript.

REFERENCES

1. Kline, D. L.: The Purification and Crystallization of Plasminogen (Profibrinolysin), J. Biol. Chem. **204:** 949, 1953.
2. Christensen, L. R.: Methods for Measuring the Activity of Components of the Streptococcal Fibrinolytic System, and Streptococcal Desoxyribonuclease, J. Clin. Invest. **28:** 163, 1949.
3. Bayne, G. M.: Personal communication.
4. Todd, M., Panter, G., Wright, I. S.: Deficiency of One or More Factors of Unusual Interest, Thrombosis et Diathesis Haemorrhagica: In press.
5. Owren, P. A.: A Quantitative One-Stage Method for the Assay of Prothrombin, Scandinav. J. Clin. & Lab. Invest. **1:** 81, 1949.
6. Adamis, D., Sise, H. S., and Kimball, D. M.: The Proconvertin Test: A Simplified Method and Its Application to the Study of Anticoagulant Processes, J. LAB. & CLIN. MED. **47:** 320, 1956.
7. Bell, W. N., and Alton, H. G.: A Brain Extract as a Substitute for Platelet Suspension in the Thromboplastin Generation Test, Nature, London **174:** 880, 1954.
8. Tocantins, L. M., editor: The Coagulation of Blood, New York, 1955, Grune & Stratton, Inc., p. 94.
9. Ratnoff, O. D., and Colopy, J. E.: A Familial Hemorrhagic Trait Associated With a Deficiency of a Clot-Promoting Fraction of Plasma, J. Clin. Invest. **34:** 602, 1955.
10. Quick, A. J.: The Determination of Prothrombin, *in* Transactions of the First Conference on Blood Clotting and Allied Problems of the Josiah Macy, Jr., Foundation, 1948, p. 170.
11. Seibert, R. H., Margolius, A., and Ratnoff, O. D.: Observations on Hemophilia, Parahemophilia, and Coexistent Hemophilia and Parahemophilia, J. LAB. & CLIN. MED. **52:** 449, 1958.
12. Sise, H. S., Lavelle, S. M., and Becker, R.: A Method for Assay of Stuart Factor, Proc. Soc. Exper. Biol. & Med. **96:** 662, 1957.
13. Owren, P. A., and Aas, K.: The Control of Dicoumerol Therapy and the Quantitative Determination of Prothrombin and Proconvertin, Scandinav. J. Clin. & Lab. Invest. **3:** 301, 1951.
14. Alagille, D., and Soulier, J. P.: Action des enzymes proteolytiques sur le sang total "in vitro." Modifications des factuers de coagulation et du complement, Semaine hôp. Paris **32:** 7, 355, 1956.
15. Lewis, J. H., Howe, A. C., and Ferguson, J. H.: Thrombin Formation: Effects of Lysin (Fibrinolysin, Plasmin) on Prothrombin, Ac-Globulin, and Tissue Thromboplastin, J. Clin. Invest. **28:** 1507, 1949.
16. Ratnoff, O. D.: Studies on a Proteolytic Enzyme in Human Plasma. IX. Fibrinogen and Fibrin as Substrates for the Proteolytic Enzyme of Plasma, J. Clin. Invest. **32:** 473, 1953.
17. Niewiarowski, S.: Effect of Plasminogen on the Clotting of Euglobulin, Odbitka z Czasopism Acta physiol. **3:** 231, 1953.
18. Mullertz, S.: The Action of Plasmin on Fibrin and Fibrinogen in Blood, Acta physiol. scandinav. **28:** 29, 1953.
19. Sherry, S.: The Participation of Fibrin in the Activation of the Fibrinolytic Enzyme of Plasma, J. Clin. Invest. **33:** 1054, 1954.
20. Seegers, W. H., and Loomis, E. C.: Prothrombin and Fibrinolysin, Science **104:** 461, 1946.
21. Niewiarowski, S., and Latallo, Z.: Influence of Plasmin on the First Coagulation Stage, Bull. Acad. polonaise sc. **5:** 219, 1957.

LABORATORY METHODS

EXAMINATION OF AN INFRARED METHOD FOR DETERMINATION OF RATIO OF COPROPORPHYRIN ISOMERS I AND III

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Investigation of an infrared spectrophotometric method for distinguishing coproporphyrin I and III tetramethylesters reveals characteristic differences in wavelengths of between 8 and 9 μ. However with samples deposited from solution nonpermanence of spectral features limits the precision of analyzing mixtures to about 10 to 15 per cent. Evidence is presented which indicates that this variability arises from gradual change in coproporphyrin III from an initially amorphous solid to a more stable crystalline state.

DETERMINATION of the type I/III isomer composition of coproporphyrins has been important in investigations of the Waldenström porphyrin and in characterization of excreted porphyrins.[1, 2] Analytically the coproporphyrin isomers are characterized by differences in melting point, in solubility, and in their x-ray and infrared spectra. Chromatographic[3, 4] and fluorescence quenching[5] methods have been developed. Infrared differentiation, despite lower sensitivity, has gained limited use with purified samples.[6, 7]

For the present work pure crystalline coproporphyrin methyl esters I and III were available, and accurate blending of stock solutions provided calibration mixtures. Nevertheless it was impossible to achieve anything approaching the spectroscopic precision to be expected of a simple binary determination. This failure led to the studies reported in this paper.

METHODS AND APPARATUS

Infrared instrumentation comprised a Perkin-Elmer 12-C with model 85 reflecting microscope, modified for linear wavelength recording at level energy input.[8] Samples in chloroform were concentrated until a few microliters deposited a dense spot upon transfer to a silver chloride slide. An alternative preparation employed potassium bromide micropellets.[9]

Crystalline samples of the four coproporphyrin isomers were available. Samples of synthetic isomers II, III, and IV were kindly provided by Dr. S. F. MacDonald.* Dr. MacDonald's synthetic III was identical with our analytic material from the residue of

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diphtheria bacillus cultures, after harvest of the toxin.* Isomer I was of natural origin, isolated from feces of patients with erythropoietic porphyria.

RESULTS

The infrared spectra of crystals of the four coproporphyrin isomers pressed into potassium bromide are shown in Fig. 1. These spectra of isomers I and III are identical with those published previously by Falk and Willis.[10] The greatest differences occur at 7.5 to 9.0 μ and at 14 to 15 μ. These differences are not large, and in fact their possible origin in impurities was suggested by earlier investigators.[11] However, by ignoring isomers II and IV, a I/III binary determination appears possible on the basis of inverse intensity of absorption of these isomers at 8.00 and 8.37 μ.[6, 7]

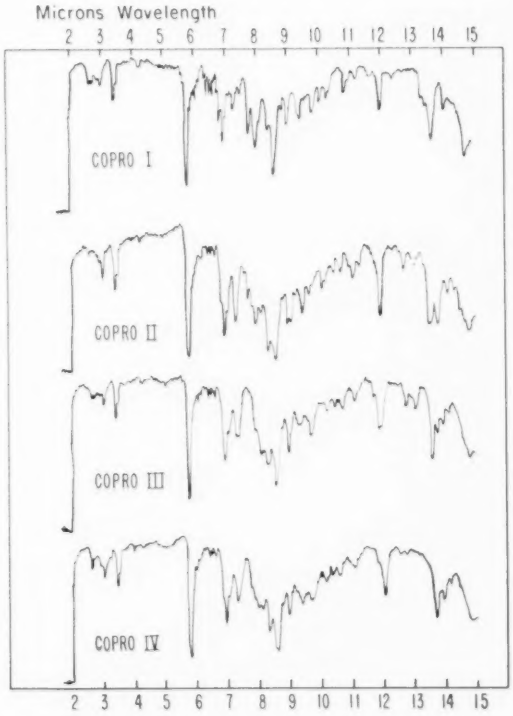

Fig. 1.—Infrared spectra of coproporphyrin isomers (methyl esters) crystals in potassium bromide pellets.

Because of limited material a practical infrared method for porphyrins will generally involve samples deposited as spots from solution, rather than crystalline material. Examination of the partial spectra *A* and *B* in Fig. 2 reveals that the coproporphyrin III spectrum of a spot from chloroform differs considerably from that of dry crystals, both in the analytic region near 8 μ and more markedly near 14 μ. The 12 μ band also loses its doublet appearance.

*Isolated by Dr. S. Schwartz and M. Keprios from material provided through the courtesy of Lederle Division of American Cyanamid Co.

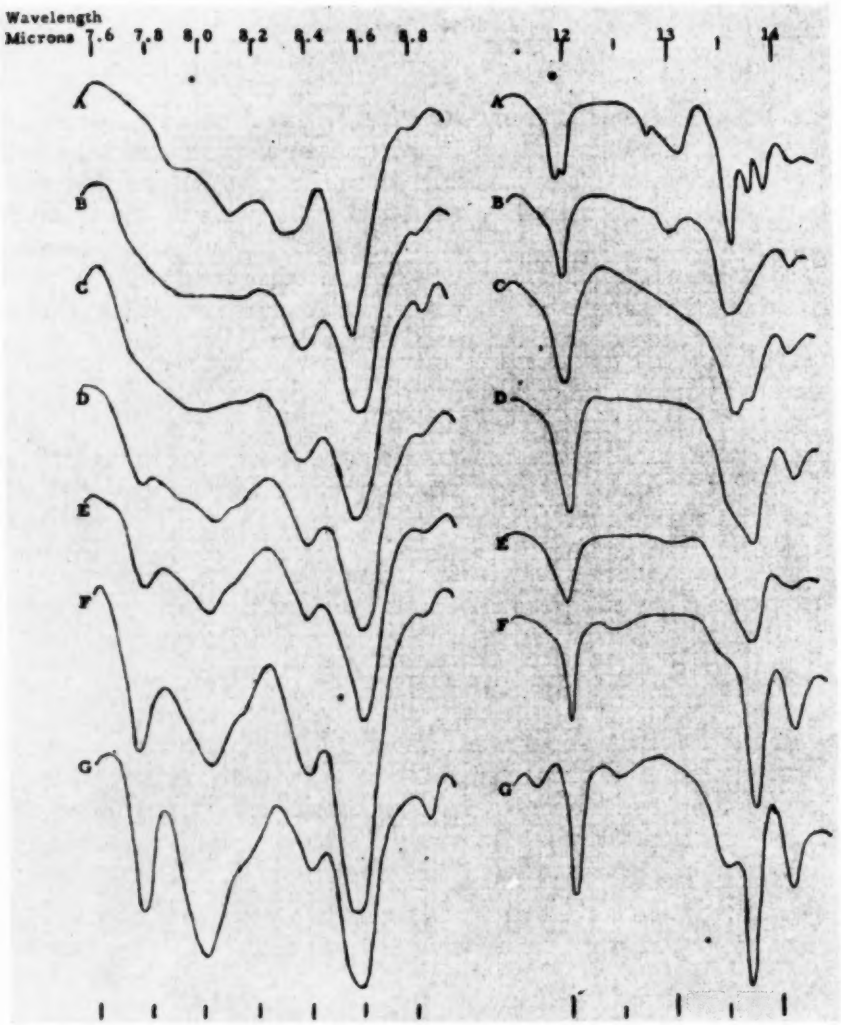

Fig. 2.—Amorphous effects in coproporphyrin III spectrum. *A*, Crystals. *B*, Spot from chloroform. *C*, Resolidified melted crystals. *D*, Spot from chloroform after one hour. *E*, Spot from chloroform after 20 hours. *F*, Spot from chloroform after 100° C. for 5 minutes. *G*, Coproporphyrin I crystals.

Spectrum *B* similarly results if the chloroform solution is prepared in a pressed potassium bromide pellet, and also if the solvent is acetone. By contrast, all deposits of isomer I from solution give the same spectrum as dry crystals.

The glassy appearance of isomer III spots from solution, which is in contrast to the grainy deposits of isomer I, suggests that the isomer III deposits are considerably less crystalline, and that curve *B* is that of *amorphous* coproporphyrin III. This conclusion is supported by the similarity of spectrum *B* with spectrum *C* of melted crystals just after resolidification.

In Fig. 3 spectra are shown for a range of I/III isomer mixtures, run as spots from chloroform after about 20 minutes' standing. The relative 8.00 to 8.37 μ band strengths afford a clear indication of the varying isomer composition, which is quantitatively expressed by the function,

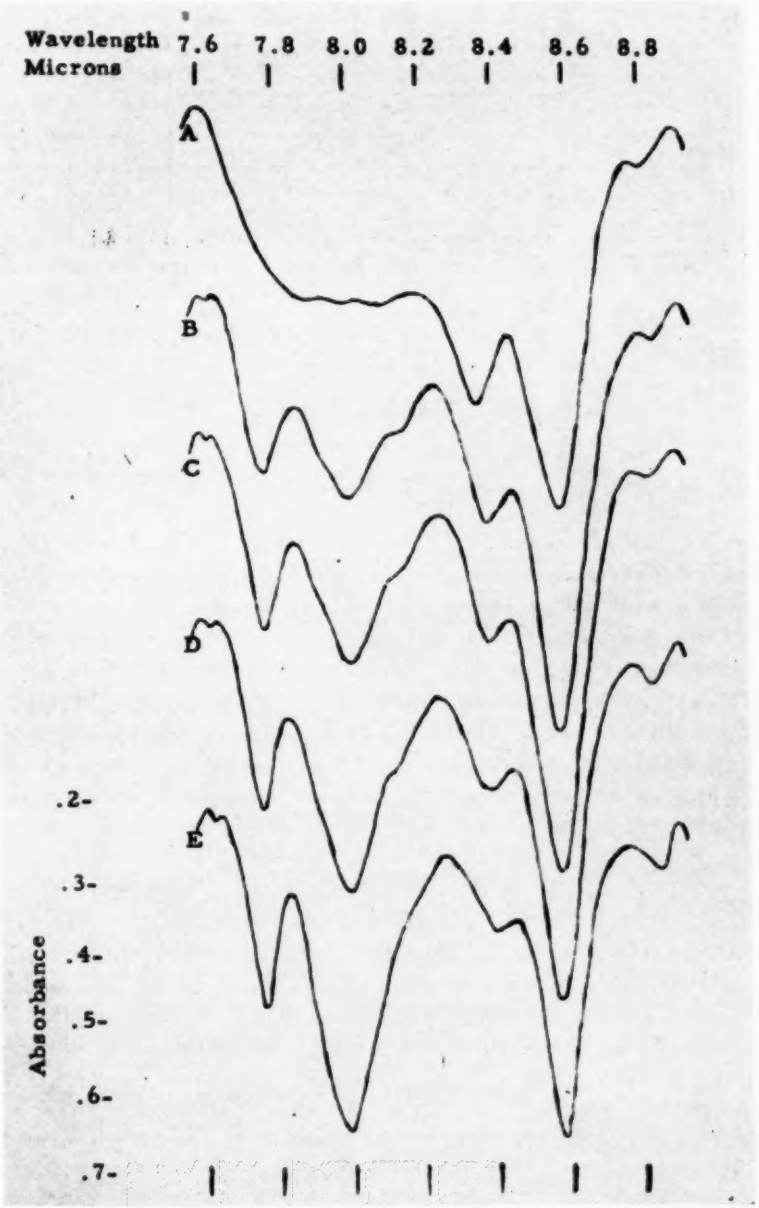

Fig. 3.—Spectra of coproporphyrin I/III mixtures, spots from chloroform after 20 to 30 minutes. *A*, Pure III. *B*, Seventy-five per cent III. *C*, Fifty per cent III. *D*, Twenty-five per cent III. *E*, Pure I.

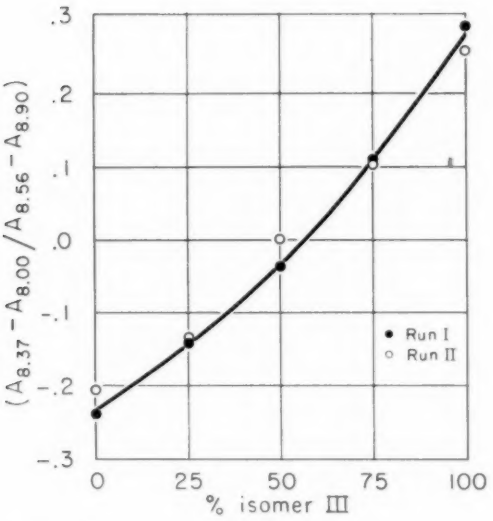

Fig. 4.—Calibration data for coproporphyrin I/III mixtures. Samples from solution pressed into potassium bromide pellets.

$$\frac{A_{8.37} - A_{8.00}}{A_{8.56} - A_{8.90}}$$

Here the A's are absorbances at the indicated wavelengths, and the function expresses the difference in absorption at the two analytic wavelengths *relative to* the 8.56 μ band which is common to both samples and is simply proportional to total sample. Such a ratio is necessary because there is no experimental control over sample thickness. Typical calibration data for two sets of known mixtures are shown in Fig. 4. The scatter in results is typical. The possibility that the spectral changes near 8 μ are not wholly compositional in origin but are also mixed with amorphous effects will be discussed in the following section. Entirely comparable data are obtained when samples are run as potassium bromide pellets.

DISCUSSION

One aspect of the amorphous character of coproporphyrin III spots which has serious analytic implications is the nonpermanence of spectral features near 8 μ. A transition toward increasing crystallinity occurs on standing and is accompanied by the sequence of spectral changes *D*, *E*, and *F* of Fig. 2. In one hour the amorphous III features are replaced by "pseudo I" features; and a few minutes in an oven at 100° C. practically duplicates in curve *F* the pure isomer I spectrum (curve *G*) near 8 μ. An analyst would report high isomer I contents from these spectra. Proof that isomer I is absent was evident in the typical behavior as type III isomer on chromatographing a portion of the 24 hour spot by the Chu-Green method.[4] That no compositional change had occurred was also shown by immediate return to the amorphous III spectrum when any older spot was redissolved in chloroform and freshly redeposited.

Spectra of mixtures relatively rich in isomer III also change, gradually taking on the appearance of higher isomer I content. Moreover, the lower the isomer III content, the greater is the spectral permanence, and, incidentally, the more granular is the spot. This suggests that spots of mixtures are initially amorphous roughly in proportion to the isomer III content; it is largely this amorphous:crystalline ratio which is being measured.

Another result of amorphism as a spectroscopic variable is a high degree of inconsistency in calibration data. Identical samples leave deposits from solutions with different degrees of crystallinity on different occasions, and the resulting spectra look different, although the compositions are the same. This has happened erratically with repeat runs on the same mixtures. Results such as these contribute most to the scatter of calibration points in Fig. 4. In earlier work with spots on silver chloride the scatter in repeat calibrations occasionally amounted to compositional differences of nearly 25 per cent but closer reproducibility has been obtained in later work with potassium bromide pellets. Typical analytic results are given in Table I for a set of prepared mixtures submitted as unknowns.

TABLE I. ANALYTIC RESULTS ON PREPARED COPROPORPHYRIN MIXTURES

SAMPLE	% III PREPARED	RUN	ABSORBANCE FUNCTION	% III FOUND
1	65	a	0.019	60
		b	−0.004	55
2	10	a	−0.176	15
		b	−0.176	15
3	40	a	−0.053	45
		b	−0.064	42
4	90	a	0.184	88
		b	0.121	78
5	20	a	−0.129	27
		b	−0.123	28

In most of the work a 20 to 30 minute period between spot deposition and spectral measurement was adhered to, since experiments in which the same spot was rerun 10, 20, 40, and 60 minutes after deposition, showed negligible spectral alteration during the first 40 minutes. In later work with potassium bromide pellets spectral measurements were repeated every half hour up to 3 hours and showed a non-diminishing progression of changes in the "pseudo I" direction. Therefore the method which has been followed, of running spectra before the onset of change, is undoubtedly best.

SUMMARY AND CONCLUSIONS

The spectral region 8 to 8.5 μ in the coproporphyrin methyl esters shows characteristic changes with I/III isomer content, but the accuracy of ± 10 per cent for a simple binary determination is less than desirable. The chief difficulty stems from the use of solid samples deposited from solution, a process which introduces a crystallinity : amorphous variable to which these absorption bands

are sensitive and which defies precise experimental control. Application of the infrared method requires 20 mcg. or more of sample of sufficient purity to be free from other absorption in the spectral region. Low precision, large sample requirement, and particularly the necessity of a high degree of preliminary purification greatly restrict application of the method. By contrast, fluorescence quenching analysis[5] has a reported accuracy of 3 per cent; both fluorescence and chromatographic techniques require only a few micrograms of sample. Infrared analysis may be useful as an independent, confirmatory check on other determinations and undoubtedly is valuable for characterization of nearly pure coproporphyrin fractions which have been isolated in large quantity.

We are indebted to Mrs. V. H. Swensen for chromatographic purification of normal isomers I and III and for preparation of known I/III mixtures.

REFERENCES

1. Watson, C. J., Schwartz, S., and Hawkinson, V.: Studies of the Uroporphyrins. II. Further Studies of the Porphyrins of the Urine, Feces, Bile, and Liver in Cases of Porphyria, With Particular Reference to a Waldenström Type Porphyrin Behaving as an Entity on the Tswett Column, J. Biol. Chem. **157:** 345, 1945.
2. Watson, C. J., and Berg, M.: Studies of the Uroporphyrins. IV. Reexamination of a Crucial Waldenström Porphyrin, J. Biol. Chem. **214:** 537, 1955.
3. Falk, J. E., and Benson, A.: Separation of Uroporphyrin Esters I and III by Paper Chromatography, Biochem. J. **55:** 101, 1953.
4. Chu, T. C., Green, A. A., and Chu, E. J.: The Chromatography of Methyl Esters of Porphyrins, J. Biol. Chem. **190:** 643, 1951.
5. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J.: A Micromethod for the Quantitative Determination of the Urinary Coproporphyrin Isomers (I and III), J. Biol. Chem. **168:** 133, 1947.
6. Berg, M., and Watson, C. J.: Studies of the Coproporphyrins. VII. Comparative Evaluation of Isomer Analysis by Paper Chromatography, Fluorescence Quenching, X-Ray Crystal Powder Pattern, and Infrared Spectrum, Abst. 169, September Meeting of the American Chemical Society, Minneapolis, 1955.
7. Watson, C. J., Berg, M., Hawkinson, V., and Bossenmaier, I.: Studies of the Uro-Porphyrins. VI. Isomer Composition of the "Waldenström Porphyrins; Effect of Heat and the Significance of Porphyrinogen, Clin. Chem. **6:** 71, 1960.
8. Dinsmore, H. L.: Infrared Microspectra of Biochemical Substances. Part I. Modification of Perkin-Elmer 12-C Spectrometer With Microscope for Linear Wavelength Recording at Level Energy Input, Spectrochimica Acta **1959:** 1025, 1960.
9. Dinsmore, H. L., and Edmondson, P. R.: Infrared Microspectra of Biochemical Substances. Part II. Pressed KBr Micropellets for Use With Reflecting Microscope, Spectrochimica Acta **1959:** 1032, 1960.
10. Falk, J. E., and Willis, J. B.: The Infrared Spectra of Porphyrins and Their Iron Complexes, Australian J. Sc. Res., sA **4:** 579, 1951.
11. Gray, C. H., Neuberger, A., and Sneath, P. H. A.: Studies in Congenital Porphyria. II. Incorporation of 15N in the Stercobilin in the Normal and in the Porphyric, Biochem. J. **47:** 87, 1950.

A MICROMETHOD FOR THE DETERMINATION OF PROTHROMBIN ACTIVITY

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A micromethod for the prothrombin assay of capillary blood of patients from whom venous blood cannot be obtained is described. The blood is drawn, immediately mixed with a solution of fibrinogen which contains an optimum amount of anticoagulant, and the assay is carried out. The method requires the use of standard equipment, and gives an excellent correlation with an accepted method.

THE routine control of anticoagulant therapy has been done for a number of years utilizing the one stage prothrombin determination of Quick[1] or the Link-Shapiro modification.[2] However, occasions arise in which it is virtually impossible to obtain a clean venipuncture and draw the required amount of blood before the initial stages of blood clotting have proceeded to a point which will affect the prothrombin assay. This is particularly true of infants, very obese persons, and patients who have been under anticoagulant therapy for extended periods of time. In such cases there is a definite need for a routine micromethod for prothrombin determinations which will give results that can be correlated with the regular method. A number of such modifications have been proposed in the last few years. Ulin and Gollub[3] described a method which was carried out on a watch glass at room temperature. Stein[4] proposed a test with whole capillary blood. A method for the estimation of prothrombin activity reported by Manchester and Rabkin[5] was performed on a glass slide. Fresh and co-workers[6] used a modified micromethod in which they diluted whole blood with a buffered anticoagulant. They concluded that the blood cells did not interfere with the accuracy of the determination. The satisfactory use of whole blood for the determination of prothrombin activity in a micromethod has been previously reported by Quick.[7] In order to eliminate possible objections to the use of whole blood in the assay of prothrombin activity, some investigators have devised methods of separating plasma from small amounts of whole blood. Sanduzzi[8] described a method whereby she obtained plasma from 0.2 ml. of whole blood, and Ashley[9] outlined methods of obtaining plasma from children for coagulation studies.

Some of the methods reported require the use of special glassware or equipment which is not readily available, and other methods do not regulate the

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temperature of the reaction. It was the purpose of this study to obtain a method for the determination of prothrombin activity in small amounts of blood which utilizes the same equipment as that required in the usual one stage method.

MATERIALS AND METHOD

Materials used were: pipettes graduated to contain 0.05 ml.; micro pipettes, certified, graduated to contain 0.10 and 0.20 ml.; Pyrex test tubes with rim, 10 by 75 mm. Fibrinogen-oxalate solution: A solution of fibrinogen which contained 300 mg. per 100 ml. coagulable protein in 0.02M sodium oxalate. (Fibrinogen from Warner-Chilcott Laboratories reconstituted with 0.02M sodium oxalate was found satisfactory for this purpose.) Thromboplastin-calcium chloride: A standardized thromboplastin suspension in 0.0125M calcium chloride. (Simplastin was used in these tests.)

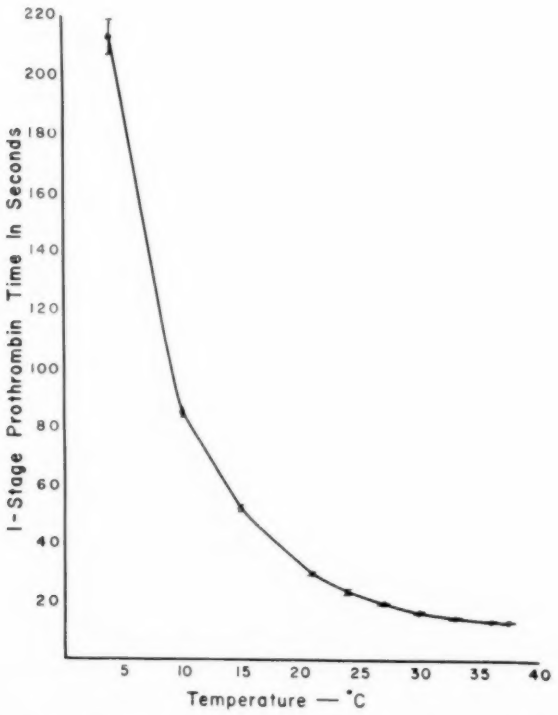

Fig. 1.—The effect of temperature upon the one stage prothrombin assay.

One stage prothrombin determination set-up: A water bath maintained at a temperature of 37.5° C. and a timer operated by a foot treadle were used.

A clean puncture was made in the finger (the ear lobe and heel were also used) with a sterile disposable lancet. The first drop of blood was wiped away and exactly 0.05 ml. of blood was drawn into a pipette and immediately blown into a small test tube which contained 0.05 ml. of oxalated fibrinogen solution. The solution served both as an anticoagulant and as a source of additional fibrin which made the clot more readily detectable. The mixture of blood and fibrinogen was drawn into the pipette and expelled; this process was repeated several times to ensure proper mixing and removal of the blood which adhered to the inner wall of the pipette. The tube was placed in the water bath at 37.5° C. As soon as the proper

temperature was reached, exactly 0.20 ml. of thromboplastin-calcium chloride mixture was blown in, and the prothrombin activity was determined in the usual manner.

Determinations of prothrombin times by the micromethod were carried out at Morristown Memorial Hospital on blood from patients who were receiving anticoagulant therapy. At the same time that the capillary blood was drawn, venous blood was taken, and the prothrombin determination was done in the usual manner.[2]

In order to establish the effect of temperature, prothrombin determinations were carried out by the one stage method at various temperatures. The prothrombin activity of normal plasma at different temperatures was plotted (Fig. 1).

RESULTS

Fig. 2 shows the values of 306 prothrombin times obtained by the regular one stage method compared with those obtained by the micro–whole blood method.

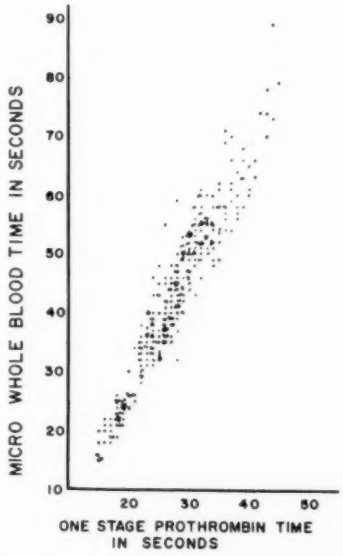

Fig. 2.—A comparison of the micro–whole blood prothrombin times with the one stage method.

The data obtained were subjected to statistical analysis by Neil R. Stasilli of the Warner-Lambert Research Institute. The prothrombin determinations performed by the regular one stage method were grouped according to time in seconds and the means and standard errors of the corresponding groups of times for the micro–whole blood method were calculated. The results are shown in Table I. Except for one standard error of ± 8 seconds (where the number of tests compared was 2), the remaining 28 groups gave errors of ± 2.9 seconds or less. It was concluded that there was a highly significant correlation between the two methods.

When the 29 group means (Y) of the times of the micro–whole blood method were plotted against the corresponding times obtained by the regular method

TABLE I. MEANS AND STANDARD ERRORS OF MICRO–WHOLE BLOOD PROTHROMBIN TIMES COMPARED WITH TIMES OF ONE STAGE PLASMA METHOD

NO. OF DETERMINATIONS	PLASMA METHOD	MICRO–WHOLE BLOOD METHOD	NO. OF DETERMINATIONS	PLASMA METHOD	MICRO–WHOLE BLOOD METHOD
6	15	16.7 ± 0.8*	16	31	52.1 ± 1.0
4	16	20.3 ± 0.9	19	32	54.1 ± 1.0
6	17	19.8 ± 0.6	10	33	54.2 ± 0.9
14	18	22.9 ± 0.5	11	34	53.3 ± 1.1
11	19	23.6 ± 0.4	8	35	55.8 ± 1.6
5	20	26.4 ± 1.0	7	36	60.4 ± 2.5
3	21	28.3 ± 2.9	6	37	62.0 ± 2.3
17	22	32.2 ± 0.7	5	38	59.0 ± 1.4
13	23	35.7 ± 0.8	5	39	63.6 ± 1.7
19	24	36.7 ± 0.9	4	40	63.8 ± 2.1
14	25	37.4 ± 1.4	2	41	64.5 ± 1.6
20	26	39.7 ± 1.1	1	42	74.0
16	27	41.1 ± 0.9	3	43	74.0 ± 2.3
25	28	43.6 ± 1.0	2	44	81.0 ± 8.0
16	29	46.9 ± 1.1	1	45	78.0
17	30	49.7 ± 0.8			

All times in seconds.

*± S. E. $= \sqrt{\frac{d^2}{N\,(N-1)}}$

(X) on linear coordinate paper, Y appeared as a linear function of X. Accordingly, a straight line was fitted to the weighted data by the method of least squares. The formula for the best fitting straight line was calculated to be as follows: $Y = 2.1\,X - 14.9$.

A comparison of results of the two methods and the daily anticoagulant dosage of typical cases is shown in Fig. 3.

DISCUSSION

It is not our intention to present a replacement of the standard one stage prothrombin determination. The control of anticoagulant therapy must be done by competent workers in laboratories with proper equipment. As our study of the effect of the variation of temperature shows, a method which is carried out at "room temperature" must be subject to elaborate control or potentially dangerous results might be obtained. It was our experience that it was necessary to dilute small volumes of blood in some manner so that the reaction with thromboplastin and calcium ions could be carried out conveniently. If this dilution was not made with a solution which contained fibrinogen, we found our correlation between the two tests to be unreliable. It should be emphasized that the fibrinogen which is used should be free from citrate ions in order to maintain the proper concentration of calcium ions. If the fibrinogen does contain citrate and additional calcium salts are added to compensate for the citrate, the increased ionic strength may affect the prothrombin time.[10] For this reason it would be necessary to dialyze citrate salts which contain fibrinogen against 0.85 per cent sodium chloride solution until the fibrinogen solution is free of citrate ions.

When the test is carried out under proper conditions, it is satisfactory for use in the control of anticoagulant therapy in those cases in which it is impossible

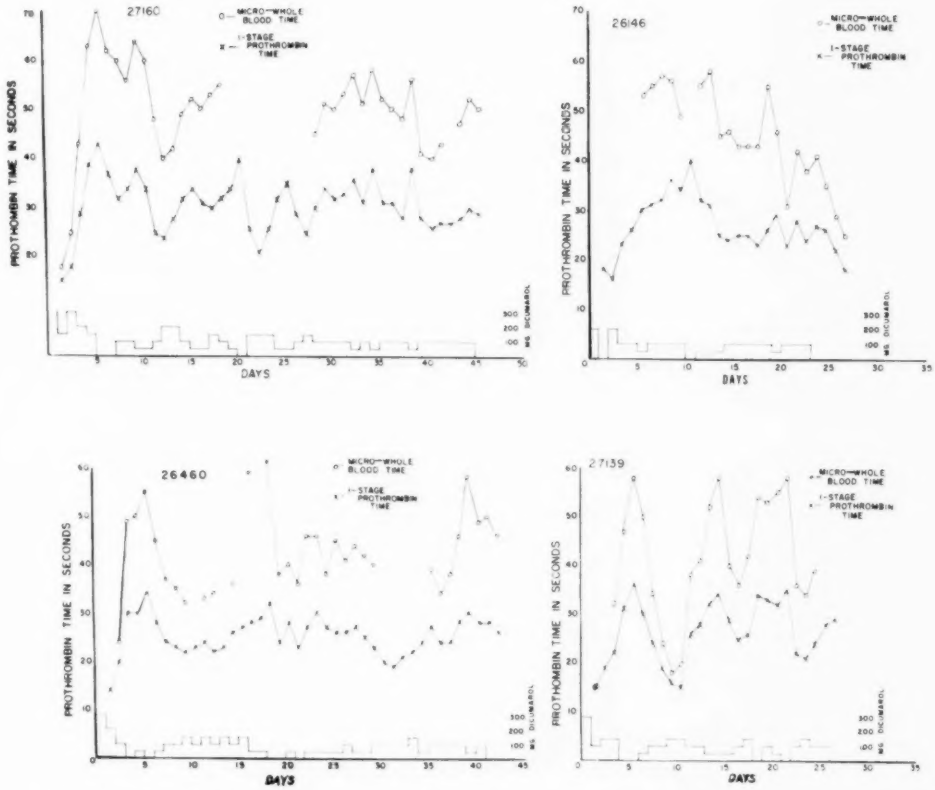

Fig. 3.—A comparison of daily prothrombin times obtained by the use of the micro–whole blood method and the one stage method.

to obtain venous blood. However, it is necessary for the workers in each laboratory who use the test to obtain their own correlation between the micro–whole blood method and the one they are presently using.

SUMMARY

A laboratory method for prothrombin assay of patients from which venous blood cannot be obtained is described. It is carried out with capillary whole blood and the standard equipment used in the one stage prothrombin tests. Excellent correlation with an accepted method was obtained.

We wish to express our appreciation to Mr. Elmer Bobst for his encouragement and support of this project.

REFERENCES

1. Quick, A. J., Stanley-Brown, M., and Bancroft, F. W.: A Study of the Coagulation Defect in Hemophilia and in Jaundice, Am. J. M. Sc. **190**: 501, 1935.
2. Shapiro, S., and Weiner, M.: Coagulation, Thrombosis and Dicumarol, New York, 1949, Brooklyn Medical Press, Inc.
3. Ulin, A. W., and Gollub, S.: New, Rapid, and Practical Micromethods for Prothrombin Determination, J. LAB. & CLIN. MED. **50**: 323, 1957.

4. Stein, H. B., and Wallace, B. V.: The Prothrombin Level in Patients on Dicumarol Therapy; a Simplified Capillary Technique, South African M. J. **26**: 353, 1952.
5. Manchester, B., and Rabkin, B.: The Control of Dicumarol Therapy in Myocardial Infarction by a Simple Blood Prothrombin Test, Circulation **10**: 691, 1954.
6. Fresh, J. W., Ferguson, J. H., Stanley, C., Morgan, F. M., and Lewis, J. H.: Blood Prothrombin, Proconvertin and Proaccelerin in Normal Infancy: Questionable Relationships to Vitamin K, Pediatrics **19**: 241, 1957.
7. Quick,, A. J.: Determination of Prothrombin, Proc. Soc. Exper. Biol. & Med. **42**: 788, 1939.
8. Sanduzzi, J.: A Micro Technique for Prothrombin Time, Am. J. M. Technol. **17**: 290, 1951.
9. Ashley, D. J. B.: Methods for the Investigation of the Coagulation Mechanism in Small Children, J. Clin. Path. **11**: 399, 1958.
10. Phillips, G. E., Weiner, M., Gilman, L., and Lenahan, J. G.: Nonspecific Factors in Blood Coagulation, J. LAB. & CLIN. MED. **46**: 641, 1955.